DESCRIPTION

INSECT RESISTANT PLANTS

Cross-Reference to Related Applications

This application is a continuation of 08/151,615, filed November 12, 1993; which is a division of Serial No. 07/713,624, filed June 10, 1991; which is a file-wrapper-continuation of Serial No. 07/260,574, filed October 20, 1988; which was a continuation-in-part of Serial No. 06/848,733, filed April 4, 1986; which was a continuation-in-part of the first filed application in this chain, Serial No. 06/535,354, filed September 26, 1983, through which the benefit of priority is hereby claimed pursuant to 35 U.S.C. §120.

Field of the Invention

The present invention is in the fields of genetic engineering, plant husbandry, and bacterial bio-affecting compositions, especially those derived from the genus *Bacillus*.

Background of the Invention

Insecticidal Protein. *Bacillus thuringiensis*, a species of bacteria closely related to *B. cereus*, forms a proteinaceous crystalline inclusion during sporulation. This crystal is parasporal, forming within the cell at the end opposite from the developing spore. The crystal protein, often referred to as the δ-endotoxin, has two forms: a nontoxic protoxin of approximate molecular weight (MW) of 130 kilodaltons (kD), and a toxin having an approximate MW of 67 kD. The crystal contains the protoxin protein which is activated in the gut of larvae of a number of insect species. Klowden, M.J. *et al.* (1983) *Appl. Environ. Microbiol.* 46:312-315, have shown solubilized protoxin from *B. thuringiensis* var. *israelensis* is toxic to *Aedes aegypti* adults. During activation, the protoxin is cleaved into two polypeptides, one or both

of which are toxic. *In vivo*, the crystal is activated by being solubilized and converted to toxic form by the alkalinity and proteases of the gut. *In vivo* the protoxin may be solubilized by extremely high pH (e.g., pH 12), by reducing agents under moderately basic conditions (e.g., pH 10), or by strong denaturants (guanidium, urea) under neutral conditions (pH 7), and once solubilized, may be activated by the action of the protease trypsin. The crystal protein is reported to be antigenically related to proteins within both the spore coat and the vegetative cell wall. Carbohydrate is not involved in the toxic properties of the protein.

B. thuringiensis and its crystalline endotoxin are useful because the crystal protein is an insecticidal protein known to be poisonous to the larvae of over a hundred species of insects, most commonly those from the orders Lepidoptera and Diptera. Insects susceptible to the action of the *B. thuringiensis* crystal protein include, but need not be limited to, those listed in Table 1. Many of these insect species are economically important pests. Plants which can be protected by application of the crystal protein include, but need not be limited to, those listed in Table 2. Different varieties of *B. thuringiensis*, which include, but need not be limited to, those listed in Table 3, have different host ranges (Faust, R.M. *et al.* [1982] in *Genetic Engineering in the Plant Sciences*, Panapolous, N.J. (ed.), pp. 225-254); this probably reflects the toxicity of a given crystal protein in a particular host. The crystal protein is highly specific to insects; in over two decades of commercial application of sporulated *B. thuringiensis* cells to crops and ornamentals, there has been no known case of effects to plants or non-insect animals. The efficacy and safety of the endotoxin have been reviewed by Faust, R.M. *et al.*, *supra*. Other useful reviews include those by Fast, P.G. (1981) in *Microbial Control of Pests and Plant Diseases*, 1970-1980, Burges, H.D. (ed.), pp. 223-248; and Huber, H.E., P. Luthy (1981) in *Pathogenesis of Invertebrate Microbial Diseases*, Davidson, E.W. (ed.), pp. 209-234.

The crystal protein gene usually can be found on one of several large plasmids that have been found in *Bacillus thuringiensis*, though in some strains it may be

located on the chromosome (Kronstad, J.W. *et al.* [1983] *J. Bacteriol.* 154:419-428). Several of the genes have been cloned into plasmids that can grow in *E. coli.* Whiteley's group (Whiteley, H.R. *et al.* [1982] in *Molecular Cloning and Gene Regulation in Bacilli*, Ganesan, A.T. *et al.* (eds.), pp. 131-144; Schnepf, H.E., H.R. Whiteley [1981] *Proc. Natl. Acad. Sci. USA* 78:2893-2897; and European patent application 63,949) reported the cloning of the toxin from *B. thuringiensis* var. *kurstaki* strains HD-1-Dipel and HD-73, using the enzymes *Sau*3AI (under partial digest conditions) and *Bgl*II, respectively, to insert large gene-bearing fragments having approximate sizes of 12 kilobasepairs (kbp) and 16 kbp into the *Bam*HI site of the *E. coli* plasmid vector pBR322. The HD-1 crystal protein was observed to be located on a 6.6 kbp *Hin*dIII fragment. Crystal protein from the HD-1-Dipel gene which was toxic to larvae, immunologically identifiable, and the same size as the authentic protoxin, was observed to be produced by transformed *E. coli* cells containing pBR322 clones or subclones. This indicated that the *Bacillus* gene was transcribed, probably from its own promoter, and translated in *E. coli.* Additionally, this suggests that the toxic activity of the protein product is independent of the location of its synthesis. That the gene was expressed when the fragment containing it was inserted into the vector in either orientation suggests that transcription was controlled by its own promoter. The transcriptional and translational start sites, as well as the deduced sequence for the amino-terminal 333 amino acids of the HD-1-Dipel protoxin, have been determined by nucleic acid sequence (Wong, H.C. *et al.* [1983] *J. Biol. Chem.* 258:1960-1967). The insecticidal gene was found to have the expected bacterial ribosome binding and translational start (ATG) sites along with commonly found sequences at -10 and -35 (relative to the 5′-end of the mRNA) that are involved in initiation of transcription in bacteria such as *B. subtilis.* Klier, A. *et al.* (1982) *EMBO J.* 1:791-799, have reported the cloning of the crystal protein gene from *B. thuringiensis* strain *berliner* 1715 in pBR322. Using the enzyme *Bam*HI, a large 14 kbp fragment carrying the crystal protein gene was moved into the vector pHV33, which can replicate in both *E. coli* and *Bacillus.* In both *E. coli* and sporulating *B.*

subtilis, the pHV33-based clone directed the synthesis of full-size (130 kD) protoxin which formed cytoplasmic inclusion bodies and reacted with antibodies prepared against authentic protoxin. Extracts of *E. coli* cells harboring the pBR322 or pHV33-based plasmids were toxic to larvae. In further work, Klier, A. *et al.* (1983) *Nucl. Acids Res.* 11:3973-3987, have transcribed the *berliner* crystal protein gene *in vitro* and have reported on the sequence of the promoter region, together with the first 11 codons of the crystal protein. The bacterial ribosome binding and translational start sites were identified. Though the expected "−10" sequence was identified, no homology to other promoters has yet been seen near −35. Held *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 77:6065-6069, reported the cloning of a crystal protein gene from the variety *kurstaki* in the phage g-based cloning vector Charon4A. *E. coli* cells infected with one of the Charon clones produced antigen that was the same size as the protoxin (130 kD) and was toxic to larvae. A 4.6 kbp *Eco*RI fragment of this Charon clone was moved into pHV33 and an *E. coli* plasmid vector, pBR328. Again, 130 kD antigenically identifiable crystal protein was produced by both *E. coli* and *B. subtilis* strains harboring the appropriate plasmids. A *B. thuringiensis* chromosomal sequence which cross-hybridized with the cloned crystal protein gene was identified in *B. thuringiensis* strains which do not produce crystal protein during sporulation.

In addition to the crystal protein, *B. thuringiensis* produces at least three other toxins. Two of them, the α-exotoxin and γ-exotoxin, are phospholipase elements that degrade lipids. *B. cereus* is also known to produce phospholipases (or lecithinases) which are toxic to insect larvae. Other bacterial enzymes which are involved in insect pathogenesis include, but need not be limited to, hyaluronidases, phosphatases, and proteases. Protease produced by *Pseudomonas aeruginosa* has been shown to have a specific affinity to proteins of *Galleria mellonella* larvae (see Lysenko, O., M. Kucera [1971] in *Microbial Control of Insects and Mites*, Burges, H.D., N.W. Hussey (eds.), pp. 205-277).

Chang, S. (1983) *Trends Biotechnol.* 1:100-101, reported that the DNA sequence of a complete HD-1 gene had been publicly presented (ref. 5 therein), and that the HD-1 toxin moiety resides in the amino-terminal 67 kD peptide.

Shuttle Vectors. Shuttle vectors, developed by Ruvkin, G.B., F.M. Ausubel (1981) *Nature* 298:85-88, provide a way to insert foreign genetic materials into position of choice in a large plasmid, virus, or genome. There are two main problems encountered when dealing with large plasmids or genomes. First, the large plasmids may have many sites for each restriction enzyme. Unique site-specific cleavage reactions are not reproducible, and multi-site cleavage reactions followed by ligation lead to great difficulties due to the scrambling of the many fragments whose order and orientation one does not want changed. Second, the transformation efficiency with large DNA plasmids is very low. Shuttle vectors allow one to overcome these difficulties by facilitating the insertion, often *in vitro*, of the foreign genetic material into a smaller plasmid, then transferring, usually by *in vivo* techniques, to the larger plasmid.

A shuttle vector consists of a DNA molecule, usually a plasmid, capable of being introduced into the ultimate recipient bacteria. It also includes a copy of the fragment of the recipient genome into which the foreign genetic material is to be inserted and a DNA segment coding for a selectable trait, which is also inserted into the recipient genome fragment. The selectable trait ("marker") is conveniently inserted by transposon mutagenesis or by restriction enzymes and ligases.

The shuttle vector can be introduced into the ultimate recipient cell, typically a bacterium of the family Rhizobiaceae (which contains the genus *Agrobacterium*), by a tri-parental mating (Ruvkin and Ausubel, *supra*), direct transfer of a self-mobilizable vector in a bi-parental mating, direct uptake of exogenous DNA by *Agrobacterium* cells ("transformation," using the conditions of Holsters, M. *et al.* [1978] *Mol. Gen. Genet.* 163:181-187), by spheroplast fusion of *Agrobacterium* with another bacterial cell, by uptake of liposome-encapsulated DNA, or by infection with a shuttle vector that is based on a virus that is capable of being packaged *in vitro*.

A tri-parental mating involves the mating of a strain containing a mobilizable plasmid, which carries genes for plasmid mobilization and conjugative transfer, with the strain containing the shuttle vector. If the shuttle vector is capable of being mobilized by the plasmid genes, the shuttle vector is transferred to the recipient cell containing the large genome, e.g., the Ti or Ri plasmids of *Agrobacterium* strains.

After the shuttle vector is introduced into the recipient cell, possible events include a double crossover with one recombinational event on either side of the marker. This event will result in transfer of a DNA segment containing the marker to the recipient genome replacing a homologous segment lacking the insert. To select for cells that have lost the original shuttle vector, the shuttle vector must be incapable of replicating in the ultimate host cell or be incompatible with an independently selectable plasmid pre-existing in the recipient cell. One common means of arranging this is to provide in the third parent another plasmid which is incompatible with the shuttle vector and which carries a different drug resistance marker. Therefore, when one selects for resistance to both drugs, the only surviving cells are those in which the marker on the shuttle vector has recombined with the recipient genome. If the shuttle vector carries an extra marker, one can then screen for and discard cells that contain plasmids resulting from a single crossover event between the shuttle vector and the recipient plasmid resulting in cointegrates in which the entire shuttle vector is integrated with the recipient plasmid. If the foreign genetic material is inserted into or adjacent to the marker that is selected for, it will also be integrated into the recipient plasmid as a result of the same double recombination. It might also be carried along when inserted into the homologous fragment at a spot not within or adjacent to the marker, but the greater the distance separating the foreign genetic material from the marker, the more likely will be a recombinational event occurring between the foreign genetic material and marker, preventing transfer of the foreign genetic material.

If the shuttle vector is used to introduce a phenotypically dominant trait (e.g., a novel expressible insecticide structural gene, but not an inactivated oncogenic T-DNA gene), one need not only rely on a double homologous recombination. The

cells resulting from a single cross-over event resulting in cointegrate plasmids can transfer the desired trait into plant cells. One may even use a variant shuttle vector having a single uninterrupted sequence of T-DNA. However, as the resulting T-DNA will now contain a tandem duplication, one must be vigilant regarding a possible rare deletion of the shuttle vector by a single homologous recombination event occurring between the two homologous sequences in either the *Agrobacterium* or plant cells.

Shuttle vectors have proved useful in manipulation of *Agrobacterium* plasmids: see Garfinkel, D.J. *et al.* (1981) *Cell* 27:143-153; Matzke, A.J.M., M.-D. Chilton (1981) *J. Mol. Appl. Genet.* 1:39-49; and Leemans, J. *et al.* (1981) *J. Mol. Appl. Genet.* 1:149-164, who referred to shuttle vectors by the term "intermediate vectors."

A recently disclosed variation of the shuttle vector system for inserting changes into large DNA molecules is the "suicide vector." In this system, as described by Puhler, A. *et al.*, U.S. Patent No. 4,626,504, and Simon, R. *et al.* (November 1983) "A Broad Host Range Mobilization System for *In Vivo* Genetic Engineering: Transposon Mutagenesis in Gram-Negative Bacteria," *Biotechnology*, pp. 784-791, the shuttle vector is incapable of being maintained within the recipient cell. This property eliminates the need to introduce an incompatible plasmid into the recipient cell in order to exclude the shuttle vector as is commonly done during a triparental mating. All vectors which do not integrate into some already present DNA effectively "commit suicide" by not being replicated. As can be done with traditional types of shuttle vectors, one may distinguish between double and single homologous recombination by screening for an antibiotic resistance gene which is not between the two regions of homology. Use of a pBR322-based suicide vector to transfer DNA sequences into a Ti plasmid has been reported by Van Haute, E. *et al.* (1983) *EMBO J.* 2:411-417, and Comai, L. *et al.* (1982) *Plant Mol. Biol.* 1:291-300.

An alternative to the use of shuttle vectors for introduction of novel DNA sequences into T-DNA by means of homologous recombination involves bacterial transposons. As described in the section Agrobacterium-Genes on the TIP Plasmids, transposons can "jump" into the T-DNA of a TIP plasmid (e.g., see Garfinkel, D.J.

et al. [1981] *Cell* 27:143-153). Should the transposon be modified *in vitro* by the insertion of the novel sequence, that novel DNA can be transferred into the TIP plasmid's T-DNA by the transposon. The TIP can then transfer the novel DNA/transposon/T-DNA combination to a plant cell where it will be stably integrated.

Agrobacterium – Overview. Included within the Gram-negative bacterial family Rhizobiaceae in the genus *Agrobacterium* are the species *A. tumefaciens* and *A. rhizogenes*. These species are respectively the causal agents of crown gall disease and hairy root disease of plants. Crown gall is characterized by the growth of a gall of dedifferentiated tissue. Hairy root is a teratoma characterized by inappropriate induction of roots in infected tissue. In both diseases, the inappropriately growing plant tissue usually produces one or more amino acid derivatives, known as opines, not normally produced by the plant, which are catabolized by the infecting bacteria. Known opines have been classified into three main families whose type members are octopine, nopaline, and agropine. The cells of inappropriately growing tissues can be grown in culture and, under appropriate conditions, be regenerated into whole plants that retain certain transformed phenotypes.

Virulent strains of *Agrobacterium* harbor large plasmids known as Ti (tumor-inducing) plasmids in *A. tumefaciens* and Ri (root-inducing) plasmids in *A. rhizogenes*. Curing a strain of these plasmids results in a loss of pathogenicity. The Ti plasmid contains a region, referred to as T-DNA (transferred-DNA), which in tumors is found to be integrated into the genome of the host plant. The T-DNA encodes several transcripts. Mutational studies have shown that some of these are involved in induction of tumorous growth. Mutants in the genes for *tml*, *tmr*, *tms*, respectively, result in large tumors (in tobacco), a propensity to generate roots, and a tendency for shoot induction. The T-DNA also encodes the gene for at least one opine synthase, and the Ti plasmids are often classified by the opine which they caused to be synthesized. Each of the T-DNA genes is under control of a T-DNA promoter. The T-DNA promoters resemble eukaryotic promoters in structure, and they appear to

function only in the transformed plant cell. The Ti plasmid also carries genes outside the T-DNA region. These genes are involved in functions which include opine catabolism, oncogenicity, agrocin sensitivity, replication, and autotransfer to bacterial cells. The Ri plasmid is organized in a fashion analogous to the Ti plasmid. The set of genes and DNA sequences responsible for transforming the plant cell are hereinafter collectively referred to as the transformation-inducing principle (TIP). The designation TIP therefore includes both Ti and Ri plasmids. The integrated segment of a TIP is termed herein "T-DNA" (transferred DNA), whether derived from a Ti plasmid or a Ri plasmid.

Chilton, M.-D. (June 1983) *Sci. Amer.* 248(6):50-59, has recently provided an introductory article on the use of Ti plasmids as vectors. Recent general reviews of *Agrobacterium*-caused disease include those by Merlo, D.J. (1982) *Adv. Plant Pathol.* 1:139-178; Ream, L.W., Gordon, M.P. (1982) *Science* 218:854-859; Bevan, M.W.; M.-D. Chilton (1982) *Ann. Rev. Genet.* 16:357-384; Kahl, G., J. Schnell (1982) *Molecular Biology of Plant Tumors*; and Barton, K.A., M.-D. Chilton (1983) *Methods Enzymol.* 101:527-539.

Agrobacterium – Infection of Plant Tissues. Plant cells can be transformed by *Agrobacterium* in a number of methods known in the art which include, but are not limited to, co-cultivation of plant cells in culture with *Agrobacterium*, direct infection of a plant, fusion of plant protoplasts with *Agrobacterium* spheroplasts, direct transformation by uptake of free DNA by plant cell protoplasts, transformation of protoplasts having partly regenerated cell walls with intact bacteria, transformation of protoplasts by liposomes containing T-DNA, use of a virus to carry in the T-DNA, microinjection, and the like. Any method will suffice as long as the gene is reliably expressed and is stably transmitted through mitosis and meiosis.

The infection of plant tissue by *Agrobacterium* is a simple technique well known to those skilled in the art (for an example, see Butcher, D.N. *et al.* [1980] in *Tissue Culture Methods for Plant Pathologists*, Ingram, D.S., J.P. Helgeson (eds.), pp. 203-208). Typically, a plant is wounded by any of a number of ways, which include

cutting with a razor, puncturing with a needle, or rubbing with an abrasive. The wound is then inoculated with a solution containing tumor-inducing bacteria. An alternative to the infection of intact plants is the inoculation of pieces of tissues such as potato tuber discs (Anand, D.K., G.T Heberlein [1977] *Amer. J. Both.* 64:153:158) or segments of tobacco stems (Barton, K.A. *et al.* [1983] *Cell* 32:1033-1043. After induction, the tumors can be placed in tissue culture on media lacking phytohormones. Hormone-independent growth is typical of transformed plant tissue and is in great contrast to the usual conditions of growth of such tissue in culture (Braun, A.C. [1956] *Cancer Res.* 16:53-56).

Agrobacterium is also capable of infecting isolated cells and cells grown in culture (Marton, L. *et al.* [1979] *Nature* 277:129-131) and isolated tobacco mesophyll protoplasts. In the latter technique, after allowing time for partial regeneration of new cell walls, *Agrobacterium* cells were added to the culture for a time and then killed by the addition of antibiotics. Only those cells exposed to *A. tumefaciens* cells harboring the Ti plasmid were capable of forming calli when plated on media lacking hormone. Most calli were found to contain an enzymatic activity involved in opine anabolism. Other workers (Horsch, R.B., R.T. Fraley [18 January 1983] 15th Miami Winter Symposium) have reported transformations by co-cultivation, leading to a high rate (greater than 10%) of calli displaying hormone-independent growth, with 95% of those calli making opines. Davey, M.R. *et al.* (1980) in Ingram and Helgeson, *supra*, pp. 209-219, describe the infection of older cells that had been regenerated from protoplasts.

Plant protoplasts can be transformed by the direct uptake of TIP plasmids. Davey, M.R. *et al.* (1980) *Plant Sci. Lett.* 18:307-313, and Davey, M.R. *et al.* (1980) in Ingram and Helgeson, *supra*, were able to transform *Petunia* protoplasts with the Ti plasmid in the presence of poly-L-α-ornithine to a phenotype of opine synthesis and hormone-independent growth in culture. It was later shown (Draper, J. *et al.* [1982] *Plant and Cell Physiol* 23:451-458; Davey, M.R. *et al.* [1982] in *Plant Tissue Culture*, Fujiwara, A. (ed.), pp. 515-516) that polyethylene glycol stimulated Ti

plasmid uptake and that some T-DNA sequences were integrated into the genome. Krens, F.A. *et al.* (1982) *Nature* 296:72-74, reported similar results using polyethylene glycol followed by a calcium shock, though their data suggests that the integrated T-DNA included flanking Ti plasmid sequences.

An alternative method to obtain DNA uptake involves the use of liposomes. The preparation of DNA containing liposomes is taught by Papahadjopoulos in U.S. Patent Nos. 4,078,052 and 4,235,871. Preparations for the introduction of Ti-DNA via liposomes have been reported (Nagata, T. *et al.* [1982] in Fujiwara, *supra*, pp. 509-510; and Nagata, T. [1981] *Mol. Gen. Genet.* 184:161-165). An analogous system involves the fusion of plant and bacterial cells after removal of their cell walls. An example of this technique is the transformation of *Vinca* protoplast by *Agrobacterium* spheroplasts reported by Hasezawa, S. *et al.* (1981) *Mol. Gen. Genet.* 182:206-210. Plant protoplasts can take up cell wall delimited *Agrobacterium* cells (Hasezawa, S. *et al.* [1982] in Fujiwara, *supra*, pp. 517-518).

T-DNA can be transmitted to tissue regenerated from a fusion of two protoplasts, only one of which had been transformed (Wullens, G.J. *et al.* [1980] *Theor. Appl. Genet.* 56:203-208). As detailed in the section on Regeneration of Plants, T-DNA can pass through meiosis and be transmitted to progeny as a simple Mendelian trait.

Agrobacterium – Regeneration of Plants. Differentiated plant tissues with normal morphology have been obtained from crown gall tumors. Braun, A.C., H.N. Wood (1976) *Proc. Natl. Acad. Sci. USA* 73:496-500, grafted tobacco teratomas onto normal plants and were able to obtain normally appearing shoots which could flower. The shoots retained the ability to make opines and to grow independently of phytohormones when placed in culture. In the plants screened, these tumorous phenotypes were not observed to be transmitted to progeny, apparently being lost during meiosis (Turgeon, R. *et al.* [1976] *Proc. Natl. Acad. Sci. USA* 73:3562-3564). Plants which had spontaneously lost tumorous properties, or which were derived from teratoma seed, were initially shown to have lost all their T-DNA (Yang, F.-M. *et al.*

[1980] *In Vitro* 16:87-92; Yang, F. *et al.* [1980] *Mol. Gen. Genet.* 177:707-714; Lemmers, M. *et al.* [1980] *J. Mol. Biol.* 144:353-376). However, later work with plants that had become revertants after hormone treatment (1 mg/1 kinetin) showed that plants that had gone through meiosis, though losing T-DNA genes responsible for the transformed phenotype, could retain sequences homologous to both ends of T-DNA (Yang, F., R.B. Simpson [1981] *Proc. Natl. Acad. Sci. USA* 78:4151-4155). Wullems, G.J. *et al.* [1981] *Cell* 24:719-724, further demonstrated that genes involved in opine anabolism were capable of passing through meiosis though the plants were male sterile and that seemingly unaltered T-DNA could be inherited in a Mendelian fashion (Wullems, G.J. [1982] in Fujiwara, *supra*). Otten, L. *et al.* (1981) *Mol. Gen. Genet.* 183:209-213, used Tn7 transposon-generated Ti plasmid mutants in the *tms* (shoot-inducing) locus to create tumors which proliferated shoots. When these shoots were regenerated into plants, they were found to form self-fertile flowers. The resultant seeds germinated into plants which contained T-DNA and made opines. In further experiments, DeGreve, H. *et al.* (1982) *Nature* 300:752-755, have found that octopine synthase can be inherited as a single dominant Mendelian gene. However, the T-DNA had sustained extensive deletions of functions other than *ocs* while undergoing regeneration from callus. Similar experiments with a *tmr* (root-inducing) mutant showed that full-length T-DNA could be transmitted through meiosis to progeny, that in those progeny nopaline genes could be expressed, though at variable levels, and that co-transformed yeast alcohol dehydrogenase I gene was not expressed (Barton, K.A. *et al.* [1983] *Cell* 32:1033-1043. It now appears that regenerated tissues which lack T-DNA sequences are probably descended from untransformed cells which "contaminate" the tumor (Ooms, G. *et al.* [1982] *Cell* 30:589-597). Recent work by Binns, A.N. (1983) *Planta* 158:272-279, indicates that tumorigenic genes, in this case *tmr*, can be "shut off" during regeneration and "turned back on" by placing regenerated tissue in culture.

Roots resulting from transformation from *A. rhizogenes* have proven relatively easy to regenerate directly into plantlets (Chilton, M.-D. *et al.* [1982] *Nature* 295:432-434).

Agrobacterium – Genes on the TIP Plasmids. A number of genes have been identified within the T-DNA of the TIP plasmids. About half a dozen octopine plasmid T-DNA transcripts have been mapped (Gelvin, S.B. *et al.* [1982] *Proc. Natl. Acad. Sci. USA* 79:76-80; Willmitzer, L. *et al.* [1982] *EMBO J.* 1:139-146) and some functions have been assigned (Leemans, J. *et al.* [1982] *EMBO J.* 1:147-152). Some of these transcripts, specifically those in the region encoding *tmr* and *tms*, can also be transcribed in prokaryotic cells (Schroder, G. *et al.* [1983] *EMBO J.* 2:403-409). The four genes of an octopine type plasmid that have been well defined by transposon mutagenesis include *tms*, *tmr*, and *tml* (Garfinkel, D.J. *et al.* [1981] *Cell* 27:143-153). Ti plasmids which carry mutations in these genes respectively incite tumorous calli of *Nicotiana tabacum* which generate shoots, proliferate roots, and are larger than normal. In other hosts, mutants of these genes can induce different phenotypes (see Bevan, M.W., M.-D. Chilton [1982] *Ann. Rev. Genet.* 16:357-384). The phenotypes of *tms* and *tmr* are correlated with differences in the phytohormone levels present in the tumor. The differences in cytokinin:auxin ratios are similar to those which in culture induce shoot or root formation in untransformed callus tissue (Akiyoshi, D.E. *et al.* [1983] *Proc. Natl. Acad. Sci. USA* 80:407-411). T-DNA containing a functional gene for either *tms* or *tmr* alone, but not functional *tml* alone, can promote significant tumor growth. Promotion of shoots and roots is respectively stimulated and inhibited by functional *tml* (Ream, L.W. *et al.* [1983] *Proc. Natl. Acad. Sci. USA* 80:1660-1664). Mutations of T-DNA genes do not seem to affect the insertion of T-DNA into the plant genome (Leemans *et al.* [1982] *supra*; Ream *et al.* [1983] *supra*). The *ocs* gene encodes octopine synthase, which has been sequenced by De Greve, H. *et al.* (1982) *J. Mol. Appl. Genet.* 1:499-511. It does not contain introns (intervening sequences commonly found in eukaryotic genes which are post-transcriptionally spliced out of the messenger precursor during maturation of the mRNA). It does have sequences

that resemble a eukaryotic transcriptional signal ("TATA box") and a polyadenylation site. All of the signals necessary for expression of the *ocs* gene are found within 295 bp of the *ocs* transcriptional start site (Koncz, C. *et al.* [1983] *EMBO J.* 2:1597-1603).

Nopaline Ti plasmids encode the nopaline synthase gene (*nos*), which has been sequenced by Depicker, A. *et al.* (1982) *J. Mol. Appl. Genet.* 1:561-573. As was found with the *ocs* gene, *nos* is not interrupted by introns. It has two putative polyadenylation sites and a potential "TATA box." In contrast to *ocs*, *nos* is preceded by a sequence which may be a transcriptional signal known as a "CAT box." All of the signals necessary for expression of the *nos* gene are found within 261 bp of the *nos* transcriptional start site (Koncz, C. *et al.*, *supra*). A gene for agrocinopine synthase and genes equivalent to *tms* and *tmr* have been identified on a nopaline-type plasmid (Joos, H. *et al.* [1983] *Cell* 32:1057-1067), and a number of transcripts have been mapped (Willmitzer, L. *et al.* [1983] *Cell* 32:1045-1056). McPhersson, J.C. *et al.* (1980) *Proc. Natl. Acad. Sci. USA* 77:2666-2670, reported the *in vitro* translation of T-DNA-encoded mRNAs from crown gall tissues.

Transcription from hairy root T-DNA has also been detected (Willmitzer, L. *et al.* [1982] *Mol. Gen. Genet.* 186:16-22). Functionally, the hairy root syndrome appears to be the equivalent of a crown gall tumor incited by a Ti plasmid mutated in *tmr* (White, F.F., E.W. Nester [1980] *J. Bacteriol.* 144:710-720).

In eukaryotes, methylation (especially of cytosine residues) of DNA is correlated with transcriptional inactivation; genes that are relatively under-methylated are transcribed into mRNA. Gelvin, S.B. *et al.* (1983) *Nucl. Acids Res.* 11:159-174, has found that the T-DNA in crown gall tumors is always present in at least one unmethylated copy. That the same genome may contain numerous other copies of T-DNA which are methylated suggests that the copies of T-DNA in excess of one may be biologically inert (see also Ooms, G. *et al.* [1982] *Cell* 30:589-597).

The Ti plasmid encodes other genes which are outside of the T-DNA region and are necessary for the infection process (see Holsters, M. *et al.* [1980] *Plasmid* 3:212-230, for nopaline plasmids; and De Greve, H. *et al.* [1981] *Plasmid* 6:235-248;

Garfinkel, D.J., E.W. Nester [1980] *J. Bacteriol.* 144:732-743; and Ooms, G. [1980] *J. Bacteriol.* 144:82-91, for octopine plasmids). Most important are the *onc* genes, which, when mutated, result in Ti plasmids incapable of oncogenicity. (These loci are also known as *vir*, for virulence.) Several *onc* genes have been accurately mapped and have been found to be located in regions conserved among various Ti plasmids (Klee, H.J. *et al.* [1983] *J. Bacteriol.* 153:878-883; Iyer, V.N. *et al.* [1982] *Mol. Gen. Genet.* 188:418-424). The *onc* genes function in *trans*, being capable of causing the transformation of plant cells with T-DNA of a different plasmid type and physically located on another plasmid (Hille, J. *et al.* [1982] *Plasmid* 7:107-118); Klee, H.J. *et al.* [1982] *J. Bacteriol.* 150:327-331; de Framond, A.J. *et al.* [1983] *Biotechnol.* 1:262-269). Nopaline Ti DNA has direct repeats of about 25 base pairs immediately adjacent to the left and right borders of the T-DNA which might be involved in either excision from the Ti plasmid or integration into the host genome (Yadav, N.S. *et al.* [1982] *Proc. Natl. Acad. Sci. USA* 79:6322-6326), and a homologous sequence has been observed adjacent to an octopine T-DNA border (Simpson, R.B. *et al.* [1982] *Cell* 29:1005-1014). Opine catabolism is specified by the *occ* and *noc* genes, respectively, of octopine- and nopaline-type plasmids. The Ti plasmid also encodes functions necessary for its own reproduction including an origin of replication. Ti plasmid transcripts have been detected in *A. tumefaciens* cells by Gelvin, S.B. *et al.* (1981) *Plasmid* 6:17-29, who found that T-DNA regions were weakly transcribed along with non-T-DNA sequences. Ti plasmid-determined characteristics have been reviewed by Merlo, *supra* (see especially Table II), and Ream and Gordon, *supra*.

Agrobacterium – TIP Plasmid DNA. Different octopine-type Ti plasmids are nearly 100% homologous to each other when examined by DNA hybridization (Currier, T.C., E.W. Nester [1976] *J. Bacteriol.* 126:157-165) or restriction enzyme analysis (Sciaky, D. *et al.* [1978] *Plasmid* 1:238-253). Nopaline-type Ti plasmids have as little as 67% homology to each other (Currier and Nester, *supra*). A survey revealed that different Ri plasmids are very homologous to each other (Costantino, P. *et al.* [1981] *Plasmid* 5:170-182). Drummond, N.H., M.-D. Chilton (1978) *J.*

Bacteriol. 136:1178-1183, showed that proportionally small sections of octopine- and nopaline-type Ti plasmids were homologous to each other. These homologies were mapped in detail by Engler, G. *et al.* (1981) *J. Mol. Biol.* 152:183-208. They found that three of the four homologous regions were subdivided into three (overlapping the T-DNA), four (containing some *onc* genes), and nine (having *onc* genes) homologous sequences. The uninterrupted homology contains at least one *tra* gene (for conjugal transfer of the Ti plasmid to other bacterial cells), and genes involved in replication and incompatibility. This uninterrupted region has homology with a *Sym* plasmid (involved in symbiotic nitrogen fixation) from a species of *Rhizobium*, a different genus in the family Rhizobiaceae (Prakash, R.K. *et al.* [1982] *Plasmid* 7:271-280). The order of the four regions is not conserved, though they are all oriented in the same direction. Part of the T-DNA sequences is very highly conserved between nopaline and octopine plasmids (Chilton, M.-D. *et al.* [1978] *Nature* 275:147-149; Depicker, A. *et al.* [1978] *Nature* 275:150-153). Ri plasmids have been shown to have extensive homology among themselves, and to both octopine (White, F.F., E.W. Nester [1980] *J. Bacteriol.* 144:710-720) and nopaline (Risuleo, G. *et al.* [1982] *Plasmid* 7:45-51) Ti plasmids, primarily in regions encoding *onc* genes. Ri T-DNA contains extensive though weak homologies to T-DNA from both types of Ti plasmid (Willmitzer, L. *et al.* [1982] *Mol. Gen. Genet.* 186:16-22). Plant DNA from uninfected *Nicotiana glauca* contains sequences, referred to as cT-DNA (cellular T-DNA), that show homology to a portion of the Ri T-DNA (White, F.F. *et al.* [1983] *Nature* 301:348-350; Spano, L. *et al.* [1982] *Plant Mol. Biol.* 1:291-300). Huffman, G.A. *et al.* (1983) *J. Bacteriol.*, have mapped the region of cross-hybridization and have shown that Ri plasmid, pRiA4b, is more closely related to a pTiA6 (octopine-type) than pTiT37 (nopaline-type) and that this Ri plasmid appears to carry sequence homologous to *tms* but not *tmr*. Their results also suggested that Ri T-DNA may be discontinuous, analogous to the case with octopine T-DNA.

It has been shown that a portion of the Ti (Chilton, M.-D. *et al.* [1977] *Cell* 11:263-271) or Ri (Chilton, M.-D. [1982] *Nature* 295:432-434; White, F.F. *et al.* [1982]

Proc. Natl. Acad. Sci. USA 79:3193-3197; Willmitzer, L. [1982] *Mol. Gen. Genet.* 186:16-22) plasmid is found in the DNA of tumorous plant cells. The transferred DNA is known as T-DNA. T-DNA is integrated into the host DNA (Thomashow, M.F. *et al.* [1980] *Proc. Natl. Acad. Sci. USA* 77:6448-6452; Yadav, N.S. *et al.* [1980] *Nature* 287:458-461) in the nucleus (Nuti, M.P. *et al.* [1980] *Plant Sci. Lett.* 18:1-6; Willmitzer, L. *et al.* [1980] *Nature* 287:359-361; Chilton M.-D. *et al.* [1980] *Proc. Natl. Acad.. Sci. USA* 77:4060-4064).

Thomashow, M.F. (1980) *supra*, and Thomashow, M.F. *et al.* (1980) *Cell* 19:729-739, found the T-DNA from octopine-type Ti plasmids to have been integrated in two separate sections, TL-DNA and TR-DNA, left and right T-DNAs, respectively. The copy numbers of TR and TL can vary (Merlo, D.J. *et al.* [1980] *Mol. Gen. Genet.* 177:637-643). A core of T-DNA is highly homologous to nopaline T-DNA (Chilton *et al.* [1978] *supra*; and Depicker *et al.* [1978] *supra*), is required for tumor maintenance, is found in TL, is generally present in one copy per cell, and codes for the genes *tms*, *tmr*, and *tml*. On the other hand, TR can be totally dispensed with (De Beuckeleer, M. *et al.* [1981] *Mol. Gen. Genet.* 183:283-288; Ooms, G. *et al.* [1982] *Cell* 30:589-597), though found in a high copy number (Merlo *et al.* [1980] *supra*). Ooms, G. *et al.* (1982) *Plasmid* 7:15-29, hypothesized that TR is involved in T-DNA integration, though they find that when TR is deleted from the Ti plasmid, *A. tumefaciens* does retain some virulence. Ooms, G. *et al.* (1982) *Cell, supra*, showed that though T-DNA is occasionally deleted after integration in the plant genome, it is generally stable and that tumors containing a mixture of cells that differ in T-DNA organization are the result of multiple transformation events. The *ocs* is found in TL but can be deleted from the plant genome without loss of phenotypes related to tumorous growth. The left border of integrated TL has been observed to be composed of repeats of T-DNA sequences which are in either direct or inverted orientations (Simpson, R.B. *et al.* [1982] *Cell* 29:1005-1014).

In contrast to the situation in octopine-type tumors, nopaline T-DNA is integrated into the host genome in one continuous fragment (Lemmers, M. *et al.*

[1980] *J. Mol. Biol.* 144:353-376; Zambryski, P. *et al.* [1980] *Science* 209:1385-1391). Direct tandem repeats were observed. T-DNA of plants regenerated from teratomas had minor modifications in the border fragments of the inserted DNA (Lemmers *et al.*, *supra*). Sequence analysis of the junction between the right and left borders revealed a number of direct repeats and one inverted repeat. The latter spanned the junction (Zambryski *et al.* [1980] *supra*). The left junction has been shown to vary by at least 70 base pairs while the right junction varies no more than a single nucleotide (Zambryski, P. *et al.* [1982] *J. Mol. Appl. Genet.* 1:361-370). Left and right borders in junctions of tandem arrays were separated by spacers which could be over 130 bp. The spacers were of unknown origin and contained some T-DNA sequences. T-DNA was found to be integrated into both repeated and low copy number host sequences. H. Joos *et al.* (1983) *Cell* 32:1057-1067, have shown that virulence is not eliminated after deletion of either of the usual nopaline T-DNA borders.

Simpson *et al.* (1982) *supra*, and Zambryski *et al.* (1980) *supra*, have suggested that direct repeats in the border regions are involved in integration of T-DNA into plant DNA. That T-DNA having borders from two different Ti plasmids are less specifically integrated than are homologous borders supports this suggestion (Ooms, G. *et al.* [1982] *Plant Mol. Biol.* 1:265-276).

Yadav, N.S. *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:6322-6326, have found a *chi* site, which in the bacteriophage (Greek symbol) augments general recombination in the surrounding DNA as far as 10 kilobases away, in a nopaline Ti plasmid just outside the left end of the T-DNA. Simpson, R.B. *et al.* (1982) *Cell* 29:1005-1014, have not observed a *chi* sequence in an octopine Ti plasmid, though the possible range of action does not eliminate the possibility of one being necessary and present but outside of the region sequenced. The significance of the *chi* in the Ti plasmid is not known. If the *chi* has a function, it is probably used in *Agrobacterium* cells and not in the plants, as *chi* is not found within the T-DNA.

Agrobacterium – Manipulations of the TIP Plasmids. As detailed in the section on Shuttle Vectors, technology has been developed for the introduction of altered

DNA sequences into desired locations on a TIP plasmid. Transposons can be easily inserted using this technology (Garfinkel, D.J. *et al.* [1981] *Cell* 27:143-153). Hernalsteen, J.-P. *et al.* (1980) *Nature* 287:645-656, have shown that a DNA sequence (here a bacterial transposon) inserted into T-DNA in the Ti plasmid is transferred and integrated into the recipient plant's genome. Though insertion of foreign DNA has been done with a number of genes from different sources, to date foreign genes have not usually been expressed under control of their own promoters. Sources of these genes include alcohol dehydrogenase (*Adh*) from yeast (Barton, K.A. *et al.* [1983] *Cell* 32:1033-1043), *Adh*I (Bennetzen, J., unpublished) and zein from corn, interferon and globin from mammals, and the mammalian virus SV40 (Schell, J., unpublished). However, when the nopaline synthase gene was inserted into octopine T-DNA and transformed into plant tissue, it was found to be fully functional (Fink, C.L. [1982] M.S. thesis, University of Wisconsin-Madison). The gene encoding phaseolin, the storage protein found in seeds of the bean *Phaseolus vulgaris* L., has been transferred into and expressed in sunflower tumors. This latter work constitutes the first example of a transferred plant gene being expressed under control of its own promoter in foreign plant tissue. Transcription started and stopped at the correct positions, and introns were post-transcriptionally processed properly (Hall, T.C. *et al.*, U.S. application Serial No. 06/485,613, which is hereby incorporated by reference). Hosters, M. *et al.* (1982) *Mol. Gen. Genet.* 185:283-289, have shown that a bacterial transposon (Tn7) inserted into T-DNA could be recovered in a fully functional and seemingly unchanged form after integration into a plant genome.

Deletions can be generated in a TIP plasmid by several methods. Shuttle vectors can be used to introduce deletions constructed by standard recombinant DNA techniques (Cohen and Boyer, U.S. Patent No. 4,237,224). Deletions with one predetermined end can be created by the improper excision of transposons (Koekman, B.P. *et al.* [1979] *Plasmid* 2:343-357, and Ooms, G. *et al.* [1982] *Plasmid* 7:15-29). Hille, J., R. Schilperoot (1981) *Plasmid* 6:151-154, have demonstrated that deletions having both ends at predetermined positions can be generated by the use

of two transposons. The technique can also be used to construct "recombinant DNA" molecules *in vivo*.

The nopaline synthase gene has been used for insertion of DNA segments coding for drug resistance that can be used to select for transformed plant cells. In plant cells, the kanamycin resistance gene from Tn5 is not transcribed under control of its own promoter (Kemp, J.D. *et al.* [18 May 1982] Beltsville Symp. VII, Beltsville, MD [1983] in *Genetic Engineering: Applications to Agriculture*, Owens, L.D. (ed.); and Fink, C.L. [1982] *supra*). Bevan, M.W. *et al.* (1983) *Nature* 340:184-187 and Fraley, R.T. *et al.* (1983) *Proc. Natl. Acad. Sci. USA* 80:4803-4807, have inserted the kanamycin resistance gene (neomycin phosphotransferase II) from Tn5 behind (i.e., under control of) the nopaline promoter. The construction was used to transform plant cells which in culture displayed resistance to kanamycin and its analogs such as G418. Schell, J. *et al.* (18 January 1983) 15th Miami Winter Symp. (see also Marx, J.L. [1983] *Science* 219:830), reported a similar construction, in which the methotrexate resistance gene (dihydrofolate reductase) from Tn7 was placed behind the nopaline synthase promoter. Transformed cells were resistant to methotrexate. Similarly, Hererra-Estrella, L. *et al.* (1983) *Nature* 303:209-213, have obtained expression in plant cells of enzymatic activity for octopine synthase and chloramphenicol acetyltransferase, an enzyme which in bacteria confers resistance to chloramphenicol, by placing the structural genes for these two enzymes under control of *nos* promoters.

Hall, T.C. *et al.*, U.S. application Serial No. 06/485,614, which is hereby incorporated by reference, have fused the *ocs* promoter and the 5′ end of the octopine synthase structural gene to the structural gene for the bean seed protein phaseolin. A fusion protein having the amino terminus of octopine synthase and lacking the amino terminus of phaseolin was produced under control of the T-DNA promoter. The introns, which were contributed by the phaseolin sequences, were post-transcriptionally processed properly.

de Framond, A.J. *et al.* (1983) *Biotechnol.* 1:262-269, have reported on the construction of a "mini-Ti plasmid." In the nopaline T-DNA there is normally only one site cut by the restriction enzyme *Kpn*I. A mutant lacking the site was constructed and a *Kpn*I fragment, containing the entire nopaline T-DNA, was isolated. This fragment together with a kanamycin resistance gene was inserted into pRK290, thereby resulting in a plasmid which could be maintained in *A. tumefaciens* and lacked almost all non-T-DNA Ti sequences. By itself, this plasmid was not able to transform plant cells. However, when placed in an *A. tumefaciens* strain containing an octopine Ti plasmid, tumors were induced which synthesized both octopine and nopaline. The mini-Ti plasmid has also been transferred into plant cells when complemented with a Ti plasmid deleted for its own T-DNA. These results indicated that the non-T-DNA functions acted in *trans* with T-DNA, that the missing nopaline Ti plasmid functions were complemented by the octopine Ti plasmid, and that the nopaline "mini-Ti" was functional in the transformation of plant cells. A similar pair of complementing plasmids, each containing either octopine T-DNA or *onc* genes, has been constructed by Hoekema, A. *et al.* (1983) *Nature* 303:179-180.

Chilton *et al.* (18 January 1983) 15th Miami Winter Symp., also reported on the construction of a "micro-Ti" plasmid made by resectioning the mini-Ti with *Sma*I to delete essentially all of T-DNA but the nopaline synthase gene and the left and right borders. The micro-Ti was inserted into a modified pRK290 plasmid that was missing its *Sma*I site, and was employed in a manner similar to mini-Ti, with comparable results.

Brief Summary of the Invention

One object of this invention is to confer pest resistance, specifically insect resistance, to a plant. In pursuance of this goal, other objects are to stably insert a gene coding for an insecticidal protein into the genome of the plant cell, to have this gene expressed in plant tissues, for the expression to be either regulated or constitutive, and for the plant tissues to be in a normal plant. Another object is to

provide novel specialized insecticidal tissues for a plant, in particular to provide a means for producing on a normal plant, preferably a dicot, a gall which contains within its tissue an insecticidal protein. Other objects and advantages will become evident from the following description.

The invention disclosed herein provides a plant comprising a genetically modified plant cell having an insecticide structural gene introduced and expressed therein under control of a plant expressible promoter. Further, the invention provides plant tissue comprising a plant cell whose genome includes T-DNA comprising an insecticide structural gene inserted in such orientation and spacing with respect to a plant expressible promoter as to be expressible in the plant cell under control of that promoter. Also provided are novel strains of bacteria containing and replicating T-DNA, as defined herein, the T-DNA being modified to contain an inserted insecticide structural gene in such orientation and spacing with respect to a plant expressible promoter as to be expressible in a plant cell under control of said promoter. Further, the invention provides novel plasmids having the ability to replicate in *E. coli* and comprising T-DNA, and further comprising an insecticide structural gene inserted within T-DNA contained within the plasmid, in such manner as to be expressible in a plant cell under control of a plant expressible promoter. Additionally, this invention discloses novel plasmids wherein the insecticide structural gene is capable of expression in *E. coli* or *Bacillus subtilis*, and furthermore discloses strains of bacteria harboring said bacterial expression plasmids.

The invention is exemplified in one of its embodiments by the insertion of the full length structural gene of *Bacillus thuringiensis* insect toxic protein into a sub-Ti plasmid so that the toxin gene is placed under the control of T-DNA plant active regulation sequences (ORF24). The sub-Ti plasmid containing the plant expressible crystal protein gene was introduced into tobacco cells. Tobacco and tomato plants regenerated from these transformed cells were found to express crystal protein at levels measurable by enzyme-linked immunosorbent assay (ELISA) techniques, but also the leaves of these plants were found to be toxic to insect larvae.

The present invention comprises an insecticide structural gene under control of a promoter expressible in plant cells, said promoter/gene combination being inserted into a plant cell by any means known in the art. More specifically, in its preferred embodiment, the invention disclosed herein further comprises expression in plant cells of an insecticide structural gene under control of a plant expressible promoter, after introduction via T-DNA, that is to say, by inserting the insecticide structural gene into T-DNA under control of a plant expressible promoter and introducing the T-DNA containing the insert into a plant cell using known means.

The invention is useful for genetically modifying plant tissues and whole plants by inserting useful insecticide structural genes from various bacterial species or strains. Such useful insecticide structural genes include, but are not limited to, the genes coding for insecticidal proteins as defined below, especially the crystal protein of *Bacillus thuringiensis*, related proteins, and the like. Truncated and modified genes, for example as described in U.S. Patent Application No. 06/617,321, incorporated herein by reference, and synthetic genes, for example as described in U.S. Patent Application No. 06/242,482, incorporated herein by reference, may also be used. The invention is exemplified by introduction and expression of structural genes for a crystal protein from *B. thuringiensis* var. *kurstaki* HD-73 or *B. thuringiensis* var. *tenebrionis* into cotton, tobacco, potato, tomato, and maize plant cells. Once plant cells expressing an insecticide structural gene under control of a plant expressible promoter are obtained, plant tissues and whole plants can be regenerated therefrom using methods and techniques well known in the art. The regenerated plants are then reproduced by conventional means, and the introduced genes can be transferred to other strains and cultivars by conventional plant breeding techniques.

The introduction and expression of the structural gene for an insecticidal protein can be used to protect a crop from infestation with insect larvae such as hornworm (*Manduca* sp.) or European corn borer (*Ostrinia nubilalis*). Other uses of the invention exploiting the properties of other insecticide structural genes introduced into other plant species will be readily apparent to those skilled in the art. The invention in principle applies to any introduction of an insecticide structural gene into

any plant species into which foreign DNA (in the preferred embodiment, T-DNA) can be introduced and in which said DNA can remain stably replicated. In general these taxa presently include, but are not limited to, gymnosperms and dicotyledonous plants, such as sunflower (family Compositeae), tobacco (family Solanaceae), alfalfa, soybeans and other legumes (family Leguminoseae), cotton (family Malvaceae), and most vegetables as well as monocotyledonous plants. The useful plants produced by the method of this invention comprise tissues toxic to insects when ingested. Pests which may be controlled by means of the present invention and the crops that may be protected from them include, but are not limited to, those listed in Tables 1 and 2, respectively. Because of its susceptibility to a number of larvae, cotton is an ideal choice for the insertion of an insecticidal protein gene. Each of the following is a major cotton pest and is also susceptible to the *B. thuringiensis* insecticidal protein: *Heliothis zea* (cotton bollworm), *Pectinophora gossypiella* (pink bollworm), *Heliothis virescens* (tobacco budworm), *Trichoplusia ni* (cabbage looper). Application of insecticidal protein prepared from sporulating *B. thuringiensis* does not control insects such as the pink bollworm in the field because of their particular life cycles and feeding habits. A plant containing in its tissues insecticidal protein will control this recalcitrant type of insect, thus providing advantage over prior insecticidal uses of *B. thuringiensis*. By incorporation of the insecticidal protein into the tissues of a plant, the present invention additionally provides advantage over such prior uses by eliminating instances of nonuniform application and the costs of buying and applying insecticidal preparations to a field. Also, the present invention eliminates the need for careful timing of application of such preparations since small larvae are most sensitive to insecticidal protein and the protein is always present, minimizing crop damage that would otherwise result from preapplication larval foraging.

Brief Description of the Drawings

Figure 1 presents the sequence of the crystal protein gene of p123/58-10, described in Example 1.

Figure 2 presents a map of restriction sites and transcripts of the T-DNA of pTi15955.

Figure 3 is a diagram of a construction described in Example 2 of a recombinant DNA vector carrying an insecticidal structural gene under control of a plant expressible promoter.

Detailed Description of the Invention

The following definitions are provided in order to remove ambiguities to the intent or scope of their usage in the specification and claims.

T-DNA: a segment of DNA derived from the transformation-inducing principle (TIP) which becomes integrated in the plant genome. As used herein, the term includes DNA originally derived from any tumor-inducing strain of *Agrobacterium* including *A. tumefaciens* and *A. rhizogenes*, the inserted segment of the latter sometimes referred to in the prior art as R-DNA. In addition, as used herein the term T-DNA includes any alterations, modifications, mutations, substitutions, insertions, and deletions, either naturally occurring or introduced by laboratory procedures, a principal structural requirement, and limitation to such modifications being that sufficient of the right and left ends of naturally-occurring T-DNAs be present to insure the expected formation of stable integration in the transformed plant cell genome which is characteristic of T-DNA. The T-DNA may itself be a composite of segments derived from a plurality of sources, naturally occurring or synthetic. In addition, the T-DNA must contain at least one plant expressible promoter, 5′ or "upstream" from the site of insertion of the insecticide structural gene, in sufficiently complete form to control initiation of transcription and initiation of translation of an inserted insecticide structural gene. This promoter may be derived from a T-DNA gene, a plant gene, or any other gene having a promoter that is functional within a plant cell in at least one tissue and at least one developmental stage. Preferably, an insertion site will be provided "downstream" in the direction of transcription and translation initiated by the promoter (3′ to the promoter), so

located with respect to the promoter to enable an insecticide structural gene inserted therein to be expressed under control of the promoter, either directly or as a fusion protein. The T-DNA may also include a 3′-untranslated region downstream from the site of insertion of the insecticide structural gene, which may function to regulate termination of transcription, polyadenylation, and post-transcriptional RNA processing. Optionally, a fusion protein may also be formed between the insecticide structural gene and the 3′ end of the structural gene donating the 3′-untranslated region. The promoter and 3′-untranslated region elements may be derived from the same or different pre-existing genes, and may be derived from the same or different plant, T-DNA, or other sources. For example, an insecticide structural gene, as exemplified herein, could be nested between a plant gene promoter and 3′ sequence from the same gene, or it could be a construct comprising the 3′-untranslated region of one gene and the promoter of another, derived from the same or different plant species of T-DNA. The coding region of a plant gene, as defined herein, may include a cDNA copy of the structural portion of a plant gene. The promoter and 3′-untranslated regions may also include modifications, either naturally or artificially induced, and may include chemically synthesized segments.

Plant promoter: As used herein includes regulatory elements of a plant gene and may further include structural elements of a plant gene, said elements being exogenous to the genes of T-DNA itself. These include, but are not limited to, promoters of the genes for phaseolin and the small subunit of ribulose-1,5-biphosphate carboxylase. Furthermore, a plant gene promoter is a region of the gene which provides for and may regulate the initiation of transcription and the initiation of translation. Additionally, the plant structural gene sequences (the region which codes for a protein in part or in whole and which may or may not contain one or more introns) may be introduced into T-DNA. (An intron is a region of a gene transcript which is post-transcriptionally removed before the mRNA is translated.) Expression under control of a plant promoter may take the form of direct expression in which the structural gene normally controlled by the promoter is removed in part

or in whole and replaced by the inserted insecticide structural gene, a start codon being provided either as a remnant of the plant structural gene or as part of the inserted insecticide structural gene, or by fusion protein expression in which part or all of the insecticide structural gene is inserted in correct reading frame phase within the existing plant structural gene. In the latter case, the expression product is referred to as a fusion protein. The promoter segment may itself be a composite of segments derived from a plurality of sources, naturally occurring or synthetic. Sources of a plant promoter include, but are not limited to, plants listed in Table 2.

T-DNA promoter: Refers to any of the naturally occurring promoters commonly associated with T-DNA. These include, but are not limited to, promoters of the "1.6" transcript, octopine synthase gene (*ocs*), nopaline synthase gene (*nos*), *tms*, *tml*, and *tmr* genes, and may depend in part on the TIP source of the T-DNA. Expression under control of a T-DNA promoter may take the form of direct expression in which the structural gene normally controlled by the promoter is removed in part or in whole and replaced by the inserted insecticide structural gene, a start codon being provided either as a remnant of the T-DNA structural gene or as part of the inserted insecticide structural gene, or by fusion protein expression in which part or all of the plant structural gene is inserted in correct reading frame phase within the existing T-DNA structural gene. In the latter case, the expression product is referred to as a fusion protein. The promoter segment may itself be a composite of segments derived from a plurality of sources, naturally occurring or synthetic.

Plant expressible promoter: As used herein includes the definitions for T-DNA promoter and plant promoter, *supra*. However, an essential aspect of the promoter component of the present invention is that the insecticide structural gene be under control of a promoter expressible in a plant cell. Therefore, plant expressible promoter additionally refers to any promoter expressible in a plant cell which is expressed in at least one tissue during at least one developmental stage. Sources might include, but need not be limited to, plant viruses (e.g., the promoters of the 35S and 19S transcripts of cauliflower mosaic virus, CaMV), animal viruses,

non-plant eukaryotes (e.g., animals, yeast), or plastids (e.g., a chloroplast or prokaryotic promoter if the insecticide gene is to be inserted into chloroplast DNA). Properties and components of a promoter that is derived from a source that is not a plant DNA or T-DNA (e.g., "TATA boxes," ATG translational start sites, intron splicing sites, etc.) are the same as described *supra* for T-DNA promoters, and plant promoters are also included within the present definition. The promoter segment may itself be a composite of segments derived from a plurality of sources, naturally occurring or synthetic.

Insecticide structural gene: As used herein includes that portion of an insecticide gene comprising a DNA segment coding for an insecticidal protein, polypeptide, or portion thereof, possibly including a translational start codon, but lacking other functional elements of a bacterial gene that regulate initiation of transcription and initiation of translation, commonly referred to as the promoter region. (Note that in the present invention such bacterial functional elements may be present after transfer of the insecticide structural gene into T-DNA. However, because they are not functional within a plant cell, such elements are not referred to by the term "insecticide structural gene.") An insecticide structural gene may be derived in whole or in part from plasmid DNA, genomic DNA, cDNA, and chemically synthesized DNA. It is further contemplated that an insecticide structural gene may contain one or more modifications in either the coding segments or untranslated regions which could affect the biological activity or chemical structure of the expression product, the rate of expression, or the manner of expression control. Such modifications could include, but are not limited to, mutations, insertions, deletions, substitutions, and "silent" modifications that do not alter the chemical structure of the expression product but which affect intercellular localization, transport, excretion, or stability of the expression product. The structural gene may constitute an uninterrupted coding sequence or it may include one or more introns, bounded by the appropriate plant functional splice junctions, which may be obtained from synthetic or a naturally occurring source. The structural gene may be a composite of segments derived from a plurality of sources, naturally occurring or

synthetic, coding for a composite protein, the composite protein being insecticidal or being derived in part from an insecticidal protein.

Insecticidal protein: As used herein includes a bacterial protein toxic in any way to insects. This includes a protein or peptide that is directly or indirectly toxic or growth inhibitory under any circumstances to any insect. This also includes proteins that are toxic upon contact, ingestion, or respiration, where alone or in combination with other material, at any time within the life cycle of an insect, including egg, larva, pupa, nymph, and adult stages. This includes proteins toxic to insects, especially those of the families Lepidoptera and Diptera, and those of the genus *Ostrinia*, *Heliothis*, *Pectinophora*, and *Trichoplusia*, e.g., *M. sexta*, *O. nubilalis*, *H. zea*, *H. virescens*, *P. gossypiella*, and *T. ni*. Other taxa that might be chosen as targets include, but are not limited, those listed in Table 1. Examples of insecticidal proteins include, but are not limited to various varieties, listed in Table 3, of *Bacillus thuringiensis*, or in other species of *Bacillus*, e.g., *B. cereus*, *B. popilliae*, and *B. sphericus*. Genes that are used to construct or otherwise encode sequences encoding proteins toxic to insects include, but are not limited to, phospholipases, hyaluronidases, phosphatases, proteases, and the various crystal proteins of *B. thuringiensis*. The term crystal protein should be understood to refer to both the full-length protoxin and toxin forms, to toxic proteins related to the protein which is found in the crystalline inclusion bodies of *Bacillus thuringiensis*, and to artificial modifications of naturally occurring crystal proteins. Related proteins might be identified by nucleic acid or protein structural or sequence homology, immunological cross-reactivity, or cross-hybridization of nucleic acids.

Plant tissue: Includes differentiated and undifferentiated tissues of plants including, but not limited to, roots, shoots, pollen, seeds, tumor tissue, such as crown galls, and various forms of aggregations of plant cells in culture, such as embryos and calli. The plant tissue may be *in planta* or in organ, tissue, or cell culture, and may be derived from plants which include, but are not limited to, those listed in Table 2.

Plant cell: As used herein includes plant cells *in planta* and plant cells and protoplasts in culture, and may be derived from plants which include, but are not limited to, those listed in Table 2.

Production of a genetically modified plant expressing an insecticide structural gene introduced via T-DNA combines the specific teachings of the present disclosure with a variety of techniques and expedients known in the art. In most instances, alternative expedients exist for each stage of the overall process. The choice of expedients depends on variables such as the choice of the basic TIP or other vector systems for the introduction and stable maintenance of the expressible insecticide structural gene, the plant species to be modified and the desired regeneration strategy, and the particular insecticide structural gene to be used, all of which present alternative process steps which those of ordinary skill are able to select and use to achieve a desired result. For instance, although the starting point for obtaining an insecticide structural gene is exemplified in the present application by DNA isolated from *B. thuringiensis* var. *kurstaki* HD-73, DNA of other insecticidal protein gene-carrying bacterial strains or recombinant DNA molecules might be substituted as long as appropriate modifications are made to the gene isolation and manipulation procedures. As novel means are developed for the controlled expression and/or stable insertion of foreign genes in plant cells, those of ordinary skill in the art will be able to select among those alternate process steps to achieve a desired result. The fundamental aspects of the invention are the nature and structure of the insecticide structural gene and its means of insertion and expression in a plant genome. The remaining steps of the preferred embodiment for obtaining a genetically modified plant include inserting the promoter/insecticide structural gene combination into T-DNA, transferring the modified T-DNA to a plant cell wherein the modified T-DNA becomes stably integrated as part of the plant cell genome, techniques for *in vitro* culture and eventual regeneration into whole plants, which may include steps for selecting and detecting transformed plant cells and steps of transferring the introduced gene from the originally transformed strain into commercially acceptable cultivars.

A principal feature of the present invention in its preferred embodiment is the construction of T-DNA having an inserted insecticide structural gene under control of a plant-expressible promoter or, most preferably, a T-DNA promoter, as these terms have been defined, *supra*. The insecticide structural gene must be inserted in correct position and orientation with respect to the desired promoter. Position has two aspects. The first relates to the side of the promoter on which the structural gene is inserted. It is known that the majority of promoters control initiation of transcription and translation in one direction only along the DNA. The region of DNA lying under promoter control is said to lie "downstream" or, alternatively, "behind" or "3′ to" the promoter. Therefore, to be controlled by the promoter, the correct position of the plant structural gene insertion must be "downstream" from the promoter. (It is recognized that a few known promoters exert bi-directional control, in which case either side of the promoter could be considered to be "downstream" therefrom.) The second aspect of position refers to the distance, in base pairs, between known functional elements of the promoter, for example, the transcription initiation site, and the translational start site of the structural gene. Substantial variation appears to exist with regard to this distance, from promoter to promoter. Therefore, the structural requirements in this regard are best described in functional terms. As a first approximation, reasonable operability can be obtained when the distance between the gene is similar to the distance between the promoter and the T-DNA gene it normally controls. Orientation refers to the directionality of the structural gene. That portion of a structural gene which ultimately codes for the amino terminus of the plant protein is termed the 5′-end of the structural gene, while that end which codes for the amino acid near the carboxyl end of the protein is termed the 3′-end of the structural gene. Correct orientation of the insecticide structural gene is with the 5′-end thereof proximal to the promoter. An additional requirement in the case of constructions leading to fusion protein expression is that the insertion of the insecticide structural gene into the promoter-donated structural gene sequence must be such that the coding sequences of the two genes are in the

same reading frame phase, a structural requirement which is well understood in the art. An exception to this requirement, of relevance to the present invention, exists in the case where an intron separates coding sequences derived from an insecticidal protein gene from the first coding segment of the insecticide structural gene. In that case, the insecticide structural gene must be provided with a splice site compatible with the upstream splice junction contributed by the noninsecticidal coding sequences, and the intron splice sites must be so positioned that the correct reading frame for the promoter-donated structural gene and the insecticide structural gene are restored in phase after the intron is removed by post-transcriptional processing. Differences in rates of expression or developmental control may be observed when a given insecticide structural gene is inserted under control of different plant expressible promoters. Different properties including, but not limited to, such properties as stability, intercellular or intracellular localization or excretion, solubility, target specificity, and other functional properties of the expressed protein itself may be observed in the case of fusion proteins depending upon the insertion site, the length and properties of the segment of T-DNA protein included within the fusion protein and mutual interactions between the components of the fusion protein that effect folded configuration thereof, all of which present numerous opportunities to manipulate and control the functional properties of the insecticidal protein product, depending upon the desired physiological properties within the plant cell, plant tissue, and whole plant.

Location of the promoter/insecticide structural gene combination insertion site is not critical as long as the transfer function sequences immediately surrounding the T-DNA borders are not disrupted, since these regions appear from prior art studies to be essential for insertion of the modified T-DNA into the plant genome. Preferred insertion sites are those which lie in areas that are most actively transcribed, in particular the *tml* gene and an area designated "1.6" lying in the *Hin*dIII-f fragment, and equivalent to transcript 24, as shown in Figure 2. The term "1.6" is used herein to designate this actively transcribed region of T-DNA. The T-DNA into which the promoter/insecticide gene combination is inserted, is obtained

from any of the TIP plasmids. The insecticide gene is inserted by standard techniques well known to those skilled in the art. The orientation of the inserted plant gene, with respect to the direction of transcription and translation of endogenous T-DNA genes is not critical; either of the two possible orientations is functional. Differences in rate of expression may be observed when a given gene is inserted at different locations within T-DNA, possibly because of such factors as DNA methylation chromatin structure. Readily detectable levels of expression of a plant promoter from the phaseolin gene have been obtained where that gene was inserted into pTi15955, an octopine-type plasmid of *A. tumefaciens* at a *Sma*I site found within the *tml* gene or a *Hpa*I site found within *tmr*.

A convenient means for inserting a promoter/insecticide structural gene combination into T-DNA involves the use of a shuttle vector, as described *supra*, having segments of T-DNA (those segments between which insertion is desired) incorporated into a plasmid capable of replicating in *E. coli*. The T-DNA segment contains a restriction site, preferably one which is unique within the shuttle vector. The insecticide structural gene can be inserted at the unique site in the T-DNA sequences, and the shuttle vector is transferred into cells of the appropriate *Agrobacterium* strain, preferably one whose T-DNA is homologous with the T-DNA segments of the shuttle vector. The transformed *Agrobacterium* strain is preferably grown under conditions which permit selection of a double-homologous recombination event which results in replacement of a pre-existing segment of the Ti plasmid with a segment of T-DNA of the shuttle vector. However, it should be noted that the present invention is not limited to the introduction of the promoter/insecticide structural gene combination into T-DNA by a double homologous recombinant mechanism; a homologous recombination event with a shuttle vector (perhaps having only a single continuous region of homology with the T-DNA) at a single site or an insertion of a promoter/gene-carrying bacterial transposon will also prove an effective means for inserting that combination into T-DNA.

Following the strategy just described, the modified T-DNA can be transferred to plant cells by any technique known in the art. For example, this transfer is most conveniently accomplished either by direct infection of plants with the novel strain containing an insecticide gene incorporated within T-DNA, or by co-cultivation of the *Agrobacterium* strain with plant cells. The former technique, direct infection, results in due course in the appearance of a tumor mass or crown gall at the site of infection. Crown gall cells can be subsequently grown in culture and, under appropriate circumstances known to those of ordinary skill in the art, regenerated into whole plants that contain the inserted T-DNA segment. Using the method of co-cultivation, a certain proportion of the plant cells are transformed, that is to say, have T-DNA transferred therein and inserted in the plant cell genome. In either case, the transformed cells must be selected or screened to distinguish them from untransformed cells. Selection is most readily accomplished by providing a selectable marker incorporated into the T-DNA in addition to the insecticide structural gene. Examples include either dihydrofolate reductase or neomycin phosphotransferase expressed under control of a nopaline synthase promoter. These markers are selected by growth in medium containing methotrexate or kanamycin, respectively, or their analogs. In addition, the T-DNA provides endogenous markers such as the gene or genes controlling hormone-independent growth of Ti-induced tumors in culture, the gene or genes controlling abnormal morphology of Ri-induced tumor roots, and genes that control resistance to toxic compounds such as amino acid analogs, such resistance being provided by an opine synthase. Screening methods well known to those skilled in the art include assays for opine production, specific hybridization to characteristic RNA or T-DNA sequences, or immunological assays for specific proteins, including ELISAs, radioimmune assays and "Western" blots. Additionally, the toxic properties of expressed insecticidal protein can be used to identify transformed tissue.

An alternative to the shuttle vector strategy involves the use of plasmids comprising T-DNA or modified T-DNA, into which an insecticide structural gene is inserted, said plasmids being capable of independent replication in an *Agrobacterium*

strain. Recent evidence reviewed in the Background indicates that the T-DNA of such plasmids can be transferred from an *Agrobacterium* strain to a plant cell, provide the *Agrobacterium* strain contains certain *trans*-acting genes whose function is to promote the transfer of T-DNA to a plant cell. Plasmids that contain T-DNA and are able to replicate independently in an *Agrobacterium* strain are herein termed "sub-TIP" plasmids. A spectrum of variations is possible in which the sub-TIP plasmids differ in the amount of T-DNA they contain. One end of the spectrum retains all of the T-DNA from the TIP plasmid, and is sometimes termed a "mini-TIP" plasmid. At the other end of the spectrum, all but the minimum amount of DNA surrounding the T-DNA border is deleted, the remaining portions being the minimum necessary to be transferable and integratable in the host cell. Such plasmids are termed "micro-TIP." Sub-TIP plasmids are advantageous in that they are small and relatively easy to manipulate directly, eliminating the need to transfer the gene to T-DNA from a shuttle vector by homologous recombination. After the desired structural gene has been inserted, they can easily be introduced directly into a plant cell containing the *trans*-acting genes that promote T-DNA transfer. Introduction into an *Agrobacterium* strain is conveniently accomplished either by transformation of the *Agrobacterium* strain or by conjugal transfer from a donor bacterial cell, the techniques for which are well-known to those of ordinary skill. For purposes of introduction of novel DNA sequences into a plant genome, TIP plasmids and sub-TIP plasmids should be considered functionally equivalent.

Although the preferred embodiment of this invention incorporates a T-DNA-based *Agrobacterium*-mediated system for incorporation of the insecticide gene into the genome of the plant which is to be made insect resistant, other means for transferring and incorporating the gene are also included within the scope of this invention. Other means for the stable incorporation of the insecticide gene into a plant genome additionally include, but are not limited to, use of vectors based on viral genomes, minichromosomes, transposons, and homologous or non-homologous recombination into plant chromosomes. Alternate forms of delivery of these vectors

into a plant cell additionally include, but are not limited to, direct uptake of nucleic acid, fusion with vector-containing liposomes, microinjection, and encapsidation in viral coat protein followed by an infection-like process. Systems based on *Agrobacterium* cells and TIPs can be used to transform dicots and gymnosperms by transfer of DNA from a bacterium to a plant cell; systems based on alternate vectors or means for vector delivery may be used to transform all gymnosperms and all angiosperms, including both monocots and dicots.

Regeneration of transformed cells and tissues is accomplished by resort to known techniques. An object of the regeneration step is to obtain a whole plant that grows and reproduces normally but which retains integrated T-DNA. The techniques of regeneration vary somewhat according to principles known in the art, depending upon the origin of the T-DNA, the nature of any modifications thereto, and the species of the transformed plant. Plant cells transformed by an Ri-type T-DNA are readily regenerated, using techniques well known to those of ordinary skill, without undue experimentation. Plant cells transformed by Ti-type T-DNA can be regenerated, in some instances, by the proper manipulation of hormone levels in culture. Preferably, however, the Ti-transformed tissue is most easily regenerated if the T-DNA has been mutated in one or both of the *tmr* and *tms* genes. Inactivation of these genes returns the hormone balance in the transformed tissue toward normal and greatly expands the ease and manipulation of the tissue's hormone levels in culture, leading to a plant that is readily regenerated because of its more normal hormone physiology. It is important to note that if the mutations in *tmr* and *tms* are introduced into T-DNA by double homologous recombination with a shuttle vector, the incorporation of the mutations must be selected in a different manner than the incorporation of the promoter/insecticide structural gene. For example, in the former instance one might select for chloramphenicol resistance while the latter selection might be for resistance to kanamycin. The inactivation of the *tms* and *tmr* loci may be accomplished by an insertion, deletion, or substitution of one or more nucleotides within the coding regions or promoters of these genes, the mutation being designed to inactivate the promoter or disrupt the structure of the protein. (The construction

of suitable mutations has been exemplified by Hall, T.C. *et al.*, U.S. patent application Serial Nos. 06/485,613 and 06/485,614.) In some instances, tumor cells are able to regenerate shoots which carry integrated T-DNA and express T-DNA genes, such as nopaline synthase, and which also express an inserted insecticide structural gene. The shoots can be maintained vegetatively by grafting to rooted plants and can develop fertile flowers. The shoots thus serve as parental plant material for normal progeny plants carrying T-DNA and expressing the insecticide structural gene inserted therein.

The genotype of the plant tissue transformed is often chosen for the ease with which its cells can be grown and regenerated in *in vitro* culture. Should a cultivar of agronomic interest be unsuitable for these manipulations, a more amenable variety is first transformed. After regeneration, the newly introduced foreign insecticidal protein gene is readily transferred to the desired agronomic cultivar by techniques well-known to those skilled in the art of plant breeding and plant genetics. Sexual crosses of transformed plants with the agronomic cultivars yielded initial hybrids. These hybrids can then be backcrossed with plants of the desired genetic background. Progeny are continuously screened and selected for the continued presence of integrated T-DNA or for the new phenotype resulting from expression of the inserted insecticidal protein gene. In this manner, after a number of rounds of backcrossing and selection, plants can be produced having a genotype essentially identical to the agronomically desired parents with the addition of the inserted insecticidal protein gene.

In an alternative method for conferring insect resistance to a crop, one may infect plants within a field which is to be protected with an *Agrobacterium* cell harboring a TIP plasmid having undisabled T-DNA which carries an expressible insecticidal protein gene. We have found that larvae will feed on crown gall tissue. When insect larvae infesting the field eat transformed tissue containing an insecticide gene, they will be affected by the insecticidal protein within that tissue. The *Agrobacterium* and TIP might additionally encode genes for insect attractants. The presence of such attractants in transformed tissue will increase the insects' preference for such tissue as a food source relative to the rest of the crop material in the field.

Examples

The following Examples utilize many techniques well known and accessible to those skilled in the arts of molecular biology and manipulation of TIPs and *Agrobacterium*; such methods are fully described in one or more of the cited references if not described in detail herein. Enzymes are obtained from commercial sources and are used according to the vendor's recommendations or other variations known to the art. Reagents, buffers, and culture conditions are also known to those in the art. Reference works containing such standard techniques include the following: Wu, R. (ed.) (1979) *Methods Enzymol.* 68; Wu, R. *et al.* (eds.) (1983) *Methods Enzymol.* 100 and 101; Grossman, L., K. Moldave (eds.) (1980) *Methods Enzymol.* 65; Miller, J.H. (1972) *Experiments in Molecular Genetics*; Davis, R. *et al.* (1980) *Advanced Bacterial Genetics*; Schleif, R.F., P.C. Wensink (1982) *Practical Methods in Molecular Biology*; and Maniatis, T. *et al.* (1982) *Molecular Cloning.* Additionally, Lathe, R.F. *et al.* (1983) *Genet. Eng.* 4:1-56, make useful comments on DNA manipulations.

Textual use of the name of the name of a restriction endonuclease in isolation, e.g., "*Bcl*I," refers to use of that enzyme in an enzymatic digestion, except in a diagram where it can refer to the site of a sequence susceptible to action of that enzyme, e.g., a restriction site. In the text, restriction sites are indicated by the additional use of the word "site," e.g., "*Bcl*I site." The additional use of the word "fragment," e.g., "*Bcl*I fragment," indicates a linear double-stranded DNA molecule having ends generated by action of the named enzyme (e.g., a restriction fragment). A phrase such as "*Bcl*I/*Sma*I fragment" indicates that the restriction fragment was generated by the action of two different enzymes, here *Bcl*I and *Sma*I, the two ends resulting from the action of different enzymes. Note that the ends will have the characteristics of being "blunt" or "sticky" (i.e., having a single-stranded protuberance capable of base-pairing with a complementary single-stranded oligonucleotide) and that the sequence of a sticky-end will be determined by the specificity of the enzyme which produces it.

In these Examples, special symbols are used to make sequences more easily understood. Sequences that code for proteins are underlined, and codons are separated with slashes (/). The positions of cuts or gaps in each strand caused by restriction endonucleases or otherwise are indicated by the placement of asterisks (*).

Plasmids, and only plasmids, are prefaced with a "p," e.g., pTi15955 or pKS-4, and strains parenthetically indicate a plasmid harbored within, e.g., *A. tumefaciens* (pTi15955) or K802(pKS-4). Table 4 provides an index useful for identifying plasmids and their interrelationships. Table 5 provides a list of deposited strains.

Example 1

The first step in developing an insect-resistant crop was to clone the insecticidal protein gene of *B. thuringiensis* var. *kurstaki* HD-73, which is on deposit with the Agricultural Research Culture Collection, Northern Regional Research Laboratory, Peoria, IL, and has NRRL number B-4488.

1.1 Cloning the *Bacillus thuringiensis* insecticidal protein gene. The 50 megadalton (MD) plasmid was enriched from HD-73 using sucrose gradient centrifugation. An HD-73 library was constructed by first digesting this plasmid with *Hin*dIII. The resulting fragments were mixed with and ligated to *Hin*dIII-linearized pRB322 (Bolivar, F. *et al.* [1978] *Gene* 2:95-113) and transformed into *E. coli* HB101. Ampicillin-resistant tetracycline-sensitive transformants were screened by digesting isolated plasmid DNA with *Hin*dIII and choosing those clones with 6.6 kilobase pair (kbp) inserts. Colonies containing plasmids p123/58-3 and p123/58-10 were selected from the HD-73 library for further analysis using an insect bioassay (see Example 8). These clones were grown in L-broth and a 250-fold concentrated cell suspension was sonicated and the extract applied to the surface of insect diet. Neonatal *Manduca sexta* (tobacco hornworm) larvae were placed on the diet for one week. Insect larvae fed extracts of strains harboring p123/58-3 or p123/58-10 did not grow, and all larvae died in 2 to 5 days. There was no apparent difference between the larvae fed these extracts and those fed insecticidal protein purified from cells of *B. thuringiensis*.

Restriction enzyme analysis of p123/58-3 and p123/58-10 showed that the two plasmids were identical except for having the 6.6 kbp *B. thuringiensis* DNA fragment inserted into the pBR322 vector in opposite orientations. Note that either of these two plasmids can be converted to the other by digestion with *Hin*dIII, religation, and transformation into HB101 followed by appropriate selection and screening steps.

p123/58-10 was used to further probe the transformants from the HD-73 plasmid library. Sixteen of the 572 colonies hybridized to the insert of clone p123/58-10 and all had the characteristic 6.6 kbp *Hin*dIII fragment. Further restriction enzyme analysis showed these clones to all be one of the two possible orientations in pBR322 of the same DNA fragment. This suggested there could be a single crystal protein gene in strain HD-73. That these clones represent the only insecticidal protein gene in HD-73 was confirmed by hybridizing labeled p123/58-10 probe to Southern blots of HD-73 plasmid DNA digested with *Hin*dIII, *Bgl*III, or *Sal*I. None of these enzymes has a restriction site in our cloned crystal protein gene. Hybridization results showed a single band of *B. thuringiensis* cellular DNA hybridized with p123/58-10 and further indicated that HD-73 has a single insecticidal crystal protein gene. We have identified a number of other clones by hybridization with a probe made from p123/58-10. Restriction mapping has shown that these clones are all identical to either p123/58-3 or p123/58-10, further supporting the conclusion that the HD-73 has a single crystal protein gene.

1.2 Immunological analysis. Analyses on the protein produced in the *E. coli* clones shows that p123/58-3 and p123/58-10 encoded protein that formed a precipitin band with antiserum to *B. thuringiensis* insecticidal protein in Ouchterlony diffusion slides. Cell extracts were analyzed on 10% sodium docecyl sulfate (SDS) polyacrylamide gels, transferred to nitrocellulose, and immunological reactions were done with antibody and ^{125}I-protein A (Western blots, Example 7). No band was found at 130 kD (kilodalton) where denatured protoxin is observed; however, a peptide of about 67 kD was seen that binds crystal protein antibody (Western blots

as done in Example 7), and was identical in size to activated toxin. This peptide accounted for approximately 1% of the total *E. coli* protein.

1.3 Sequence analysis. We compared our DNA sequence results (Figure 1), obtained by methods well known to those skilled in the art of DNA sequencing (e.g., see Maxam, A.M., W. Gilbert [1980] *Methods Enyzmol.* 65:499-560), with published sequences (see Background). The published sequences showed only partial homology with our own sequence. An open reading frame of about 2.8 kbp was observed which bounded at the 5'-end by a translational start signal (ATG) and did not stop before encountering the *Hin*dIII site at the junction between the *B. thuringiensis* DNA and the pBR322 vector. The size of the protein encoded by this open reading frame from the ATG to the *Hin*dIII site is greater than that of the 67 kD protein that we observed to be translated in *E. coli* cells but less than what is needed to encode the 130 kD native crystal protein. That the exact means of translational termination in the pBR322 encoded read-through peptide was not important was suggested by the finding that insecticidal activity was encoded by *B. thuringiensis* DNA inserts having either orientation within the pBR322 vector. Presumably the initially translated amino acid residues carboxy-terminal to the ultimate carboxy-terminus of the translated polypeptide were removed in *E. coli* by a proteolytic process similar to that which naturally activates the crystal protein.

Example 2

This example teaches the insertion of the *Bacillus thuringiensis* insecticide gene between a T-DNA gene promoter and a polyadenylation (poly(A) addition) signal, the transfer of the insecticide gene to various plant species *via* a Ti plasmid, and the regeneration of plants expressing this gene under control of the T-DNA promoter. A large part of the strategy used in this construction is diagrammed in Figure 3, which represents plasmids schematically and is not necessarily drawn to scale.

2.1 Introduction of *Bam*HI site into the insecticidal protein gene. A *Bam*HI site is introduced into the insecticidal protein gene of p123/58-10 at a location just 5'

to the start of the coding sequence. The wild-type base sequence (b) and the changed bases in an oligonucleotide primer (a) are as follow:

```
                    BamHI
(a)  5' AGATGGAG*GATCCTT ATG GAT AAC AAT 3'
(b)  ...AGATGGAG GTAACTT/ATG/GAT/AAC/...
                         Met Asp Asn Asn
```

The changed bases are the underlined ATC sequence in (a). Note that good hybridization properties are insured because only three out of 27 base pairs are changed.

p123/58-10 is digested with *Hin*dIII and is mixed with and ligated to *Hin*dIII-linearized mWB2344 RF (replicative form) DNA. The mixture is transformed into JM103, and transformed colonies are screened by plasmid isolation followed by restriction analysis for the presence of insertion of a single copy of the insecticidal protein gene-bearing fragment. Vectors containing the two possible orientations are labeled M13-Bt-A and M13-Bt-S. They have the antisense and sense strands, respectively, of the insecticide structural gene when in viral form. M13-Bt-A is hybridized with the oligonucleotide primer, 5'-AGATGGAGGA TCCTTATGGA TAACAAT-3', previously synthesized as described in Example 10.1. The oligonucleotide: M13-Bt-A hybrid is incubated with the Klenow fragment of *E. coli* DNA polymerase I, covalently closed circular DNA (cccDNA) is enriched, and the mixture is transformed into JM103. The virions produced by transformants are isolated and used to infect cells at a low multiplicity of infection. RF DNA is isolated from a number of the infected colonies and is characterized by restriction mapping. Clones derived from the mutant oligonucleotide-primed strand are identified by the presence of a novel *Bam*HI site at the 5'-end of the insecticide structural gene, and one such vector is designated M13-Bt-A(*Bam*).

M13-Bt-A(*Bam*) RF DNA is digested with *Bam*HI and *Hin*dIII, and is mixed with and ligated to a linker, synthesized as described in Example 10.1, having the following structure:

```
        HindIII          BamHI
5'AGCTAGCTGACTAG3'
    3'TCGACTGATCCTAG5'
```

Note that this linker contains translational stop signals (underlined) in all three possible reading-phases. The linkers are trimmed by digestion with *Bam*HI and an insecticide structural gene-bearing DNA fragment is purified by agarose gel electrophoresis.

2.2 Construction and modification of a promoter vehicle. The T-DNA "1.6" gene is summarized as follows:

```
                ClaI          960 bp  250 bp  ClaI  60 bp
5'...TACACCAAAT*CG/ATG/GAC/ATG/.../TGA/....AT*CGAT....AAAT
  promoter         Met Asp Met ....stop         polyadenyla-

 50 bp
AA...AAATAA...3'
tion signals
```

By removing the *Cla*I fragment, the promoter region of the "1.6" gene can be brought next to the 3'-downstream region of the gene. This 3' region includes polyadenylation signals. The resulting structure is summarized as follows:

```
             ClaI          60 bp          50 bp
5'...ATACACCAAAT*CGATAGT.......AAATAA........AAATAAAA...3'
   promoter                  polyadenylation signals
```

pKS111, which is a pRK290 clone corresponding to the T-DNA clone p403 (which encodes the "1.6" gene which was described in the Detailed Description, transcript 24 in Figure 2, see also Fink, C.F. [1982] M.S. thesis, University of Wisconsin-Madison), is digested with *Cla*I and then religated. The ligation mix is transformed into *E. coli* K802 (Wood, W.B. [1966] *J. Mol. Biol.* 16:118) and selected for tetracycline resistance. Plasmids are isolated by doing "minipreps" (plasmid preparations from small volume cell cultures), and restriction maps are obtained to prove the structure. The new vehicle, pKS-*pro*I (see Hall, T.C. *et al.*, U.S. application Serial No. 06/485,614), can be linearized by *Cla*I.

pKS-*pro*I grown in K802 was cut with *Cla*I. After converting sticky-ends to blunt-end with the Klenow fragment of *E. coli* DNA polymerase I, the DNA was mixed with and ligated to a *Bam*HI linker. The resulting mixture was digested with *Cla*I to remove religated pKS-*pro*I, and transformed into K802. Plasmids from tetracycline-resistant transformants are screened by restriction analysis, and a plasmid having the *Cla*I site at the ATG translational start replaced with a *Bam*HI site is designated pKS-*pro*I(*Bam*).

2.3 Introduction of a kanamycin resistance gene into pKS-*pro*I(*Bam*). It is advantageous to have a kanamycin resistance (*kan*) resistance gene inserted next to the promoter/insecticide gene combination so as to allow selection of double homologous recombinants after a triparental mating. The source of *kan* was pKS-4 (Example 2.5). In pKS-4 the *kan* gene is flanked on one side by a *Cla*I site. In order to remove a *kan* gene-bearing fragment from pKS-4 with *Cla*I (i.e., on a *ClaI/kan*" fragment) it is necessary to introduce a *Cla*I site into that plasmid on the opposite side of *kan* from the existing *Cla*I site. This is accomplished by converting a conveniently positioned *Bam*HI site (5'. . . G*GATCC . . . 3') to the specificity of *Cla*I (5' . . . AT*CGAT . . . 3').

pKS-4 is linearized by digestion with *Bam*HI, thereby generating sticky-ends having the following structures:

```
5'...G          GATCC...3'
3'...CCTAG          C...5'
```

The recessed ends of this structure are filled in by incubation with the Klenow fragment of *E. coli* DNA polymerase I, forming the following blunt-ends:

```
...GGATC        GATCC...
...CCTAG        CTAGG...
```

When these ends were blunt and ligated together, the resulting suture has the following sequence:

```
          ClaI
...GGAT*CG ATCC...
...CCTA GC*TAGG...
```

Note that the resulting structure is susceptible to the action of *Cla*I but not to that of *Bam*HI.

Alternatively to the above construction, one may convert the *Bam*HI site, or another conveniently located restriction site, into a *Cla*I site by use of the appropriate linkers. pKS-4 was digested with *Sma*I mixed with and ligated to *Cla*I/blunt-ended linkers having the sequence 5'-CATCGATG-3', digested with *Cla*I, religated, and transformed into K802. Plasmids isolated from transformants resistant to kanamycin were screened for presence of a novel *Cla*I site in the position formally occupied by a *Sma*I site. A *ClaI/kan* fragment can be isolated from such a plasmid. The plasmid is designated pKS4.2.

When grown in *E. coli* K802, pKS-*pro*I(*Bam)* is methylated at two remaining *Cla*I sites: one is about 145 bases from the promoter-polyadenylation junction (this is about 30 bases past the second polyadenylation site); the other is about 200 bases from the right hand p403 *Eco*RI site (see Figure 2). Methylation blocks cutting by the *Cla*I restriction endonuclease at an otherwise susceptible site. Therefore, these methylations protect these sites and effectively direct action of the *Cla*I enzyme to other sites. pKS-*pro*I(*Bam*) is transferred to and grown in *E. coli* GM33, a strain that does not methylate adenosine residues in DNA, so that the otherwise methylated *Cla*I sites can be cut. After purification of that plasmid from GM33 (pKS-*pro*I(*Bam*)), a partial digestion is done with *Cla*I and the resulting mixture is ligated with the *ClaI/kan* fragment described above. After transformation into *E. coli* K802, transformants are selected on tetracycline and kanamycin containing media. After plasmid isolation and restriction mapping, a clone having the desired construction is identified, and the plasmid found in this clone is labeled p11-83a (Figure 3).

p11-83a has a *kan* gene-bearing fragment ligated into the "middle" *Cla*I site about 30 bp past the second polyadenylation site. The *Bam*HI fragment of the insecticide gene, isolated from the modified vector constructed in Example 2.1, is now ligated into the *Bam*HI site of *Bam*HI-linearized p11-83a that has been transferred to and grown in K802 and is methylated. After transformation into K802, tetracycline

and kanamycin selection, plasmid isolation, and restriction enzyme mapping, the desired construction having the insecticide structural gene inserted between the pTi15955 "1.6" promoter and polyadenylation site is identified, and the plasmid harbored therein is labeled p11-83b (Figure 3).

2.4 Introduction of p11-83b into Ti plasmids. p11-83b is introduced into pTi15955, pTiA66 (equivalent to pTi15955 but having a nonfunctional *tms* gene), and mutants deleted in genes affecting regeneration by homologous recombination (Example 10). Tobacco plants are transformed by a system described in Example 6, and transformants are identified by Southern and Northern blots (techniques well known to those skilled in the art) with appropriate probes and by the presence of octopine and crystal protein. Transformed tobacco tissue is lethal to tobacco hornworms. Tobacco plants are regenerated from transformed cells, as described in Example 6, and entered into breeding programs. Fields of regenerated plants and their insecticidal protein-containing descendants are resistant to infestation by larvae of insects such as tobacco hornworm by virtue of the toxic effect such larvae experience when eating tissue from such plants.

2.5 Cloning and isolation of a kanamycin resistance gene. pRZ102 (Jorgenson, R.A. *et al.* [1979] *Mol. Gen. Genet.* 177:65-72), a ColE1 plasmid carrying a copy of the transposon Tn5, was digested with *Bam*HI and *Hin*dIII, mixed with pBR322 previously linearized with the same two enzymes, ligated, and transformed into K802. Plasmids isolated from transformants selected for resistance to both ampicillin and kanamycin were restriction mapped, and one having the structure shown in Figure 3 was labeled pKS-4. pKS-4 DNA may be isolated from *E. coli* C600 (pKS-4), which has been deposited as NRRL B-15394.

Example 3

This example teaches another method of inserting an expressible gene for the *B. thuringiensis* insecticidal protein into a plant genome. The shuttle vector is similar to that used by Fink, C.L. (1982) M.S. thesis, University of Wisconsin-Madison, to put

the *nos* gene into an octopine Ti plasmid. In the present invention, the protein coding sequences for *nos* are removed and replaced with an insecticidal gene before insertion into the Ti plasmid. The eventual result is an octopine-type Ti plasmid carrying an insecticide gene expressible in plant cells under control of a nopaline synthase promoter.

3.1 Moving the *nos* gene into M13mp7. pCF44 (Fink, *supra*) was digested with *Xho*I, religated to itself, and transformed back into K802. Plasmid DNA isolated from ampicillin-resistant transformants was analyzed with restriction enzymes. A plasmid having a single *Xho*I site within its Ti plasmid-derived DNA sequences was designated pCF44A. The single *Xho*I site was the result of the deletion of a DNA fragment between the two pCF44 *Xho*I sites. Deletion of this *Xho*I fragment resulted in the complete removal of two inconvenient *Cla*I sites.

pCF44 DNA was digested with *Hin*dIII and *Bam*HI, mixed with and ligated to a double-stranded circular replicative form (RF) of the single-stranded DNA vector M13mp7 which had been linearized with *Bam*HI. After transformation of the mixture into JM103 and selection of white plaques, two colonies were identified by restriction mapping after RF isolation, designated M13-1 and M13-3, contained the sense and antisense strands, respectively, when in single-stranded form.

3.2 Placement of an *Nco*I site behind the *nos* promoter. An oligonucleotide primer having the sequence 5′-AGTCTCATAC TCACTCTCAA TCCAAATAAT CTGCCATGGA T-3′ was synthesized as described in Example 10.1. This oligonucleotide was changed at the underlined base from the naturally occurring sequence at the 5′-end of the *nos* structural gene. The change resulted in the introduction of an *Nco*I site, 5′ . . . C*CATGG . . . 3′, at the ATG translational start of the *nos* gene. The oligonucleotide was hybridized to circular single-stranded M13-3 DNA isolated from virions which had been sedimented out of culture medium. The oligonucleotide:M13-3 hybrid was incubated with DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I, covalently closed circular DNA (cccDNA) was enriched, and the mixture was transformed into JM103. The virions produced by

transformants were isolated and used to infect cells at a low multiplicity of infection. RF DNA was isolated from a number of these infected colonies and characterized by restriction mapping. Clones derived from the mutant oligonucleotide-primed strand were identified by the presence of a single *Nco*I site, which allowed them to be linearized by that enzyme. The mutated clones were further characterized to localize the *Nco*I site by digestion with *Cla*I, *Bam*HI (to identify linearized molecules), and *Cla*I together with *Nco*I. The mutated M13-3 vector was labeled M13-3A/B18a.

3.3 Moving the insecticide gene into M13mp8. p123/58-10 DNA (Example 1.1) was digested with *Eco*RI and mixed with and ligated to *Eco*RI-linearized M13mp8 RF DNA. After transformation of the mixture into JM103 and selection of white plaques, two colonies having the insecticide gene-carrying fragment inserted in opposite orientations were identified by restriction mapping. They were labeled MBT14 and MBT3 and respectively had the sense and antisense strands when in single-stranded form.

3.4 Placement of an *Nco*I site at the insecticide gene translation start. An oligonucleotide primer having the sequence 5′-GAGGTAACCC ATGGATAACA AT-3′ is synthesized as described in Example 10.1. This oligonucleotide is changed at the two underlined bases from the naturally occurring sequence at the 5′-end of the insecticide structural gene. The change results in the introduction of an *Nco*I site, 5′ . . . C*CATGG . . . 3′, at the ATG translational start of the insecticide gene. The oligonucleotide is hybridized to circular single-stranded MBT3 DNA isolated from virions which had been sedimented out of culture medium. The oligonucleotide:MBT3 hybrid is incubated with DNA ligase and the Klenow fragment of *E. coli* DNA polymerase II, cccDNA is enriched, and the mixture is transformed into JM103. The virions produced by the transformants are isolated and used to infect cells at a low multiplicity of infection. RF DNA is isolated from a number of these infected colonies and characterized by restriction mapping. Clones derived from the mutant oligonucleotide-primed strand are identified by the presence of a single *Nco*I site which allows them to be linearized by that enzyme. The mutated

clone is further characterized by restriction enzyme analysis, and the presence of the mutant sequence is confirmed by sequencing. The plasmid having the desired sequence is labeled MBT3(*Nco*).

3.5 Assembly of a plant expressible insecticide gene in a shuttle vector. *Nco*I- and *Hin*dIII-digested MBT3(*Nco*) RF DNA is mixed with and ligated to a linker, synthesized as described in Example 11.1, having the following structure:

```
HindIII-end      BamHI
 5'AGCTGACTAACTAG3'
     3'CTGATTGATCCTAG5'
```

This linker encodes stop codons (underlined) in all three reading phases, and is ended by a functional *Bam*HI site and a *Hin*dIII compatible sticky-end incapable of reconstructing a *Hin*dIII site. The insecticide gene-bearing DNA fragment is then trimmed by digestion with *Nco*I and *Bam*HI and is isolated by an agarose gel electrophoresis.

pKS111-N (Fink, *supra*) is a plasmid having a *nos* gene inserted in Tn5 DNA (from pKS-4) which has a functional *kan* gene, which is itself inserted in the T-DNA of pKS111. pKS111-N is linearized with *Sst*II and digested to completion with *Bam*HI. M13-3A/B18a is digested with *Nco*I and *Sst*II and the *Sst*II/*Nco*I promoter fragment is isolated by agarose gel electrophoresis. The *Sst*II/*Nco*I promoter and *Nco*I/*Bam*HI gene fragments are mixed with and ligated to the pKS111-N *Sst*II/*Bam*HI reaction products. The ligation mixture is then transformed into *E. coli* K802. Plasmids isolated from transformants resistant to kanamycin and tetracycline are subjected to restriction enzyme analysis, and colonies harboring plasmids identical to pKS111-N, except for replacement of a 5'-portion of the *nos* gene with an insecticide structural gene, are identified. Such a plasmid is designated pKS111-NpBt.

3.6 Insertion into TIP plasmids, plant infection, and regeneration. *E. coli* K802(pKS111-NpBt) is mated with *A. tumefaciens* as described in Example 9. The *Agrobacterium* strains chose harbor TIP plasmids, based on pTi15955, containing mutations, such as those described in the Background, which facilitate regeneration. Homologous recombinants are selected as described in Example 9 and characterized

by restriction mapping. The efficacy of the construction is quickly tested by infection of sunflower stems. The resulting galls are assayed by ELISA and Western blots as described in Example 7 and by bioassay as described in Example 8. As described in Example 6, the *Agrobacterium* strains are used to infect tobacco cells which are then regenerated. The resulting plants are used as breeding stock to be crossed with various commercial varieties for which insect resistance properties are desired. Regenerated plants and fields of their insecticidal protein-containing descendants are resistant to infestation by larvae of insects such as tobacco hornworm by virtue of the toxic effect such larvae experience when eating tissue from such plants.

Example 4

This example teaches another method of inserting an expressible gene for the *B. thuringiensis* insecticidal protein into a plant genome. The strategy is similar to that described in Example 3 but differs in that a plant promoter is used instead of a T-DNA promoter. The plant gene providing the promoter is phaseolin, which has been shown to be active in species other than its source, the bean *Phaseolus vulgaris* L.

4.1 Moving the phaseolin gene into M13mp7. *Bam*HI linkers, having the sequence 5′-GGATCC-3′, are annealed to form double-stranded structures and blunt-end ligated to form concatamers. These concatamers are partially digested with *Bam*HI to expose 5′GATC . . . 3′ sticky-ends, which are compatible with sticky-ends generated by the enzymes *Bam*HI, *Bcl*I, *Mbo*I, *Sau*3AI, and *Xho*II (5′GATC . . . 3′). A Charon 24A phage clone of phaseolin, 177.4 (Sun, S.M. *et al.* [1981] *Nature* 289:37-41; Slightom, J.L. *et al.* [1983] *Proc. Natl. Acad. Sci.* USA 80:1897-1901, also designated AG-PVPh177.4) is digested with *Bgl*II and *Bam*HI, mixed with and ligated to the concatenated linkers, and completely digested with *Bam*HI to trim the linkers and expose the *Bam*HI sticky-ends; a 3.8 kbp fragment containing the phaseolin gene and 5′- and 3′-flanking sequences is isolated by agarose gel electrophoresis followed by elution. This fragment has *Bam*HI sites at either end as the *Bam*HI/*Bgl*II linkage

site is not susceptible to the action of either enzyme. The 3.8 kbp *Bgl*II/*Bam*HI fragment can also be obtained from p8.8, a pBR322-based subclone of 177.4.

The 3.8 kbp fragment is mixed with and ligated to *Bam*HI-linearized M13mp7 RF. After transformation of the mixture into JM103 and selection of white plaques, two colonies are selected after characterization by restriction and hybridization analysis of RFs and phage DNA. The viral forms of M13-3.8A and M13-3.8S are respectively found to be carrying the antisense and sense strands of the phaseolin gene.

4.2 Placement of an *Nco*I site behind the phaseolin promoter. The *Phaseolus* DNA of M13-3.8A has an *Nco*I site about 450 bp upstream from the phaseolin transcriptional start. The presence of this site will be inconvenient when one wishes to cleave the plasmid at the *Nco*I site to the introduced at the phaseolin translational start. Isolated M13-3.8A RF DNA is linearized with *Nco*I, and the 5′-protruding ends are filled in by the action of the Klenow fragment of *E. coli* DNA polymerase I. After blunt-end ligation and transformation into JM103, RF DNAs are isolated and characterized by restriction mapping. A colony is chosen which harbors a vector, labeled M13-3.8Ac, which is lacking the *Nco*I site of the *Phaseolus* DNA but is otherwise unchanged from M13-3.8A.

An oligonucleotide primer having the sequence 5′-ATACTACTCT ACCATGGTGA GAGCAAGGG-3′ is synthesized as described in Example 10.1. This oligonucleotide is changed at the underlined bases from the naturally occurring sequence at the 5′-end of the phaseolin gene. The oligonucleotide is hybridized to circular single-stranded M13-3.8Ac DNA isolated from virions which had been sedimented out of culture medium. The oligonucleotide:M13-3.8Ac hybrid is incubated with DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I, cccDNA is enriched, and the mixture is transformed into JM103. The virions produced by transformants are isolated and used to infect cells at a low multiplicity of infection. RF DNA is isolated from a number of these infected colonies and characterized by restriction mapping. Clones derived from the mutant

oligonucleotide-primed strand are identified by the presence of a novel *Nco*I site positioned at the 5′-end of the coding sequence. The mutated clones are further characterized to localize the *Nco*I site by digestion with *Cla*I and *Cla*I together with *Nco*I. The mutated M13-3.8Ac vector is labeled M13-3.8Aa.

4.3 Placement of a *Hin*dIII site at the 3′-end of the phaseolin gene. To conveniently introduce the insecticide gene into the phaseolin gene, two additional changes must be made to that phaseolin gene. The first change involves addition of *Hin*dIII site (5′ . . . A*AGCTT . . . 3′) 5′ to the polyadenylation site and near the 3′-end of the phaseolin gene. The other important change involves placing translational stop codons (e.g., TAA, TAG, or TGA, underlined below) in all three reading frames. When the oligonucleotide 5′-AGGGTGCATT TGAAGCTTGA ATAAGTAAGA ACTAAAATGC-3′ (a) is compared with the 3′-end of the phaseolin gene coding sequence (b), it can be seen to have the desired properties as follows:

```
                    HindIII
(a) 5'AGGGTGCATTTGA*AGCTTGAATAAGTAAGAACTAAAATGC3'
(b) ..AGGGTGCATTTGT GTACTGAATAAGTATGAACTAAAATGC..
mismatches:         ↑ ↑↑↑↑          ↑
```

Note also that this 38-mer has only 6 mismatches, thus insuring good hybridization properties during priming.

The oligonucleotide 5′-AGGGTGCATT TGAAGCTTGA ATAAGTAAGA ACTAAAATGC-3′, synthesized as described in Example 10.1, is hybridized to single-strand circular M13-3.8Aa DNA purified from virions isolated by centrifugation of culture medium. The oligonucleotide:M13-3.8Aa hybrid is incubated with DNA ligase and the Klenow fragment of *E. coli* DNA polymerase I, cccDNA is enriched, and the mixture is transformed into JM103. The virions produced by the transformants are isolated and used to infect cells at a low multiplicity of infection. RF DNA is isolated from a number of the infected colonies and characterized by restriction enzyme analysis. Clones derived from the mutant oligonucleotide-primed strand are identified by the presence of a *Hin*dIII site mapping at the 3′-end of the phaseolin gene, and

the presence of mutant sequences at both ends of the structural gene is confirmed by sequencing. A vector containing the desired sequences is labeled M13-3.8Ab.

4.4 Inserting the insecticide gene. MBT3(*Nco*) RF DNA is digested with *Nco*I and *Hin*dIII and is mixed with and ligated to *Nco*I- and *Hin*dIII-digested M13-3.8Ab DNA. The mixture is transformed into K802, and plasmid DNA from kanamycin- and/or tetracycline-resistant transformants is isolated and characterized by restriction enzyme analysis. A plasmid having the insecticide structural gene inserted between the phaseolin promoter and polyadenylation site is labeled M13-PpBt, and a colony harboring it is chosen.

4.5 Moving the modified phaseolin gene into a shuttle vector. pKS111-K (Fink, *supra*) has the Tn5 *kan* gene from pKS-4 inserted between the *Hin*dIII sites of pKS111 T-DNA. M13-PpBt/RF DNA is digested with *Bam*HI and mixed with and ligated to *Bam*HI-linearized pKS111-K (Fink, *supra*). Plasmids from K802 transformants resistant to kanamycin and/or tetracycline are isolated and characterized by restriction mapping. A colony is selected which harbors a plasmid, labeled pKS111-PpBt, which contains the phaseolin promoter/insecticide structural gene/polyadenylation site combination which, together with a *kan* gene, is surrounded by octopine T-DNA.

4.6 Insertion into TIP plasmids, plant infection and regeneration. *E. coli* K802(pKS111-PpBt) is mated with *A. tumefaciens* as described in Example 9. The *Agrobacterium* strains chosen harbor TIP plasmids, based on pTi15955, containing mutations, such as those described in the Background, which facilitate regeneration. Homologous recombinants are selected as described in Example 9 and characterized by restriction mapping. The efficacy of the construction is quickly tested by infection of sunflower stems. The resulting galls are assayed by ELISA and Western blots as described in Example 7 and by bioassay as described in Example 8. As described in Example 6, the *Agrobacterium* strains are used to infect tobacco cells which are then regenerated. The resulting plants are used as breeding stock to be crossed with various commercial varieties for which insect resistance properties are desired. Fields

of regenerated plants and their insecticidal protein-containing descendants are resistant to infestation by larvae of insects such as tobacco hornworm by virtue of the toxic effect such larvae experience when eating tissue from such plants.

Example 5

Regeneration in this Example involves carrot tumors incited by Ri-based TIP plasmids and is effected essentially as described by Chilton, M.-D. *et al.* (1982) *Nature* 295:423-434.

5.1 Infection with hairy root. Carrot discs are inoculated with about 10^9 bacteria in 0.1 ml of water. One to 1.5 cm segments of the ends of the roots obtained are cut off, placed on solid (1-1.5% agar) Monier medium lacking hormones (Tepfer, D.A., J.C. Tempe [1981] *Cr. Hebd. Seanc. Acad. Sci., Paris* 295:153-156), and grown at 25°C to 27°C in the dark. Cultures uncontaminated by bacteria are transferred every 2 to 3 weeks and are subcultured in Monier medium lacking hormones and agar. Transformed roots can be recognized by their aberrant morphology and selected.

5.2 Regeneration of roots to plants. The cultured root tissue described in Example 5.1 is placed on solidified (0.8% agar) Monier medium supplemented with 0.36 μM 2,4-D and 0.72 μM kinetin. After 4 weeks, the resulting callus tissue is placed in liquid Monier medium lacking hormones. During incubation at 22 to 25°C on a shaker (150 rpm) for one month, the callus disassociates into a suspension culture from which embryos differentiate, and which, when placed in Petri dishes containing Monier medium lacking hormones, develop into plantlets. These plantlets are grown in culture, and after "hardening" by exposure to atmospheres of progressively decreasing humidity, are transferred to soil in either a greenhouse or field plot.

5.3 Use of non-hairy root vectors. Ti-based vectors which do not have functional *tmr* genes are used instead of the Ri-based vectors as described by Hall, T.C. *et al.*, U.S. applications Serial Nos. 06/485,613 and 06/485,614. Construction of

suitable mutants can be done by those skilled in the art, and is reviewed in the Background.

Example 6

Regeneration in this Example involves tobacco leaves incited by a Ti-based TIP plasmid and is effected essentially as described by Barton, K.A. *et al.* (1983) *Cell* 32:1033-1043.

6.1 Infection with crown gall. Tobacco tissue is transformed using an approach utilizing inverted stem segments first described by Braun, A.C. (1956) *Canc. Res.* 16:53-56. Stems are surface sterilized with a solution of 7% commercial "CLOROX" and 80% ethanol, rinsed with sterile distilled water, cut into 1 cm segments, placed basal end up in Petri dishes containing agar-solidified MS medium (Murashige, T., F. Skoog [1962] *Physiol. Plant.* 15:473-497) lacking hormones. Inoculation is effected by puncturing the cut basal surface of the stem with a syringe needle and injecting bacteria. Stems are cultured at 25°C with 16 hours of light per day. The calli which develop are removed from the upper surface of the stem segments, are placed on solidified MS medium containing 0.2 mg/ml carbenicillin and lacking hormones, are transferred to fresh MS-carbenicillin medium three times at intervals of about a month, and are tested to ascertain whether the cultures had been rid of bacteria. The axenic tissues are maintained on solidified MS media lacking supplements under the culture conditions (25°C; 16 hr:8 hr light:dark) described above.

6.2 Culture of transformed tissue. Clones are obtained from the transformed axenic tissues as described by Binns, A., F. Meins (1979) *Planta* 145:365-369. Calli are converted into suspensions of cells by culturing in liquid MS having 0.02 mg/l naphthalene acetic acid (NAA) at 25°C for 2 or 3 days while being shaken at 135 rpm, and filtering in turn through 543 and 213 μm stainless steel meshes. The passed filtrate is concentrated, plated in 5 ml of MS medium containing 0.5% melted agar, 2.0 mg/l NAA, 0.3 mg/l kinetin, and 0.4 g/l Difco yeast extract at a density of about

8×10^3 cells/ml. Colonies reaching a diameter of about 1 mm are picked by scalpel point, placed onto and grown on solidified MS medium having 2.0 mg/l NAA, 0.3 mg/l kinetin, and about 10 μg/ml S-(2-aminoethyl)-L-cysteine (AEC). (A range of concentrations of AEC, between about 2 μg/ml and about 30 μg/ml, is tried as the exact concentration effective for selection will depend on the variety of tobacco used and the growth conditions to which the source plant and tissues derived from it are subjected.) AEC has been shown to be an agent capable of selecting tissue containing octopine synthase (Dahl, G.A., J. Tempe [1983] *Theor. Appl. Genet.* 66:233-239). Alternatively, the filtrate is plated at low density (several hundred cells per ml) on a filter paper overlaying a feeder layer of tobacco cells growing on the solidified MS/NAA/kinetin/yeast extract medium. When 1 mm colonies have formed, the entire filter paper is transferred to a Petri dish containing the solidified MS/NAA/kinetin/AEC medium. The resulting calli which do not show the effects of AEC toxicity are selected, split into pieces, and tested for other transformed phenotypes such as production of octopine and hormone-independent growth.

6.3 Regeneration of plants. Transformed clones are placed onto solidified MS medium having 0.3 mg/l kinetin, and cultured as described in Example 6.1. The shoots which form are rooted by putting them on a solid (1.0% agar) medium containing 1/10 strength MS medium salts, 0.4 mg/l thiamine, lacking sucrose and hormones, and having a pH of 7.0. Rooted plantlets are grown in culture, hardened as described in Example 5.2, and are transferred to soil in either a greenhouse or field plot. Plants are screened for retention of the transformed phenotype by methods well known to those skilled in the art, such as Southern, Northern, and dot blots with appropriate probes, octopine assays, immunological (see Example 7) or biological (Example 8) assays for presence of crystal protein.

6.4 Vectors used. Constructions described by Hall, T.C. *et al.*, U.S. application Serial Nos. 06/485,613 and 06/485,614 are suitable Ti-based vectors lacking functional *tmr* genes. The method described in Example 6.1 for infection of

inverted stem segments is often useful for the establishment of TIP-transformed plant cell lines.

Example 7

Anti-insecticidal protein antibody was produced by methods well known to those skilled in the art of immunology. "Western" blots, to detect antigens after SDS-polyacrylamide gel electrophoresis (SDS-PAGE), were done essentially as described by Legocki, R.P., D.P.S. Verma (1981) *Anal. Biochem.* 111:385-392.

Micro-ELISA assays are done using Immulon-2 type plates having 96 wells by the following steps:

7.1 Binding antibody to plates. On day one, the wells are coated with 1:1000 dilution of antibody (rabbit anti-insecticidal protein IgG) in coating buffer. 200 μl/well are incubated at 37°C for 2-4 hours. The plates are covered with "SARAN WRAP" during this incubation. Afterwards the plates are rinsed three times with phosphate-buffered saline-"TWEEN" (PBS-Tween), allowing a five-minute waiting period between each rinse step. Then 1% bovine serum albumin (BSA) is added to rinse and, after addition to the well, left to sit for 20 minutes before discarding. Rinsing is repeated five times more with PBS-Tween.

7.2 Tissue homogenization. The tissue is sliced up into small pieces and then homogenized with a polytron using 1 gm of tissue/ml PBS-Tween-2% polyvinyl pyrrolidone-40 (PBS-Tween-2% PVP-40). All samples are kept on ice before and after grinding, and standard curves were obtained. One standard curve is done in tissue homogenates, and one standard curve is also done in buffer to check the recovery of insecticidal protein from homogenized tissue or cells. Following centrifugation of the homogenized samples, 100 μl of each sample is placed in a well and left overnight at 4°C. To avoid errors, duplicates of each sample are done. The plates are sealed during incubation.

to the shuttle vector carrying strain and mobilized the shuttle vector for transfer to the *Agrobacterium*. Growth on a medium containing both streptomycin and the drug to which the shuttle vector is resistant, often either kanamycin or chloramphenicol, resulted in the selection of *Agrobacterium* cells containing shuttle vector sequences. A mating of these cells with *E. coli* (pPH1J1) resulted in the transfer of pPH1J1 to the *Agrobacterium* cells. pPH1J1 and pRK290-based shuttle vectors cannot coexist for long in the same cell. Growth on gentamycin, to which pPH1J1 carries a resistance gene, resulted in selection of cells having lost the pRK290 sequences. The only cells resistant to streptomycin, gentamycin, and kanamycin are those which have Ti plasmids that have undergone double-homologous recombination with the shuttle vector and now carry the desired construction. pRK290 and pRK2013 were disclosed by Ditta, G. *et al.* (1980) *Proc. Natl. Acad. Sci. USA* 77:7347-7357, and pPH1J1 by Hirsh, P.R. (1978) Thesis, Univ. E. Anglia.

Example 10

This Example describes techniques for the synthesis and use of synthetic oligonucleotides. Other useful references can be found in the list of works cited in the section introductory to these Examples.

10.1 Oligonucleotide synthesis. The techniques for chemical synthesis of DNA fragments used in these Examples utilize a number of techniques well known to those skilled in the art of DNA synthesis. The modification of nucleosides is described by Schaller, H. *et al.* (1963) *J. Amer. Chem. Soc.* 85:3821-3827. The preparation of deoxynucleoside phosphoramidites is described by Beaucage, S.L., M.H. Caruthers (1981) *Tetrahedron Lett.* 22:1859. Preparation of solid phase resin is described by Adams, S.P. *et al.* (1983) *J. Amer. Chem. Soc.* Hybridization procedures useful during the formation of double-stranded synthetic linkers are described by Rossi, J.J. *et al.* (1982) *J. Biol. Chem.* 257:9226-9229.

10.2 Use for oligonucleotides. Use of synthetic oligonucleotides to reconstruct a deleted segment of a gene has been exemplified by Hall *et al.*, U.S. application

Serial No. 06/485,614. Use of synthetic oligonucleotides to link otherwise incompatible restriction site sticky-ends has been exemplified by Hall *et al.*, U.S. application Serial No. 06/485,614 and is well known to those skilled in the art of recombinant DNA manipulations.

10.3 Oligonucleotide-directed mutagenesis. General methods of directed mutagenesis have been reviewed recently by Shortle, D. *et al.* (1981) *Ann. Rev. Genet.* 15:265-294. Of special utility in manipulation of genes is oligonucleotide-directed site-specific mutagenesis, reviewed recently by Zoller, M.J., M. Smith (1983) *Methods Enzymol.* 100:468-500; Smith, M., S. Gillam (1981) in *Genetic Engineering: Principles and Methods*, Vol. 3, Setlow, J.K., A. Hollaender (eds.); Smith, M. (1982) *Trends in Biochem.* 7:440-442. This technique permits the change of one or more base pairs in a DNA sequence or the introduction of small insertions or deletions. Recent examples of use of oligonucleotide-directed mutagenesis include Zoller, M.J., M. Smith (1983) *supra*; Zoller, M.J., M. Smith (1982) *Nucl. Acids Res.* 10:6487-6500; Dalbadie-McFarland, G. *et al.* (1982) *Proc. Natl. Acad. Sci. USA* 79:6409-6413; Simons, G.F.M. *et al.* (1982) *Nucl. Acids Res.* 10:821-832; and Hutchinson, C.A., III *et al.* (1978) *J. Biol. Chem.* 253:6551-6560. Useful M13-based vectors (e.g., mWB2344) have been reported by Barnes, W.M. *et al.* (1983) *Meth. Enzymol.* 101:98-122; and Barnes, W.M., M. Bevan (1983) *Nucl. Acids Res.* 11:349-368.

The sequence to be modified usually is moved into a single-stranded bacteriophage vector, here one derived from M13, by standard techniques well known to those in the art. The vector DNA is generally in the double-stranded replicative form (RF), as the single-stranded viral form cannot ordinarily be "cut and spliced" by restriction enzymes and ligases. After *in vitro* ligation of the fragment into the RF, transformation into a suitable host, and production of single-stranded DNA (ssDNA) as part of the life cycle of the vector, ssDNA is isolated from phage particles and hybridized to an oligonucleotide having sufficient length and sequence homology to hybridize to the vector in the appropriate location. The oligonucleotide should have the sequence desired as an end product and otherwise differ in no way from the

sequence to be changed. Once a hybrid is formed comprising a ssDNA circle base-paired to the oligonucleotide carrying the mutant sequence, the oligonucleotide primes synthesis of a complementary strand of DNA by the Klenow fragment of *E. coli* DNA polymerase I, a polymerase lacking a 5′-to-3′ exonuclease activity. The vector is optionally incubated with DNA ligase, and the polymerase and ligase reactions may be done simultaneously. Preferentially covalently closed-circular double-stranded DNA (cccDNA) molecules can be selected before transformation by techniques which include alkaline sucrose gradient centrifugation, extraction with phenol under alkaline conditions, and incubation with S1 nuclease. The vector can now be transformed into an appropriate bacterial host cell. Virus particles from this initial infection are isolated and used to form plaques by infecting a lawn of bacteria. In cases where one is changing a restriction site, one may readily screen RFs by restriction enzyme analysis. One may also screen by hybridization under carefully selected conditions using the synthetic mutant oligonucleotide primer as a probe, or by DNA sequencing. When a clone containing the desired change has been isolated, one may manipulate the now mutant DNA as desired using techniques well known to those skilled in the art.

Example 11

This Example teaches isolation of a clone having the 3′-end of the insecticide gene carried by p123/58-10, and the reconstruction of a full-length HD-73 crystal protein gene.

11.1 Cloning of an insecticide gene 3′-end. Immunodetection of electrophoretically separated peptides on protein blots and DNA sequencing showed that p123/58-10 and p123/58-3 each contained a partial protoxin gene. To reconstruct a complete protoxin gene, flanking DNA restriction sites were identified by Southern blots of restriction digests, a well-known technique, and overlapping clones were selected from a *Pst*I library made from 50 MD plasmid-enriched DNA as follows: 50 MD plasmid DNA, enriched by sucrose gradient centrifugation as above, was

digested to completion with *Pst*I, mixed with and ligated to *Pst*I-linearized pBR322, and transformed into HB101. Tetracycline-resistant transformants were screened essentially as described by Benton, W.D., R.W. Davis (1977) *Science* 196:180-182, using a probe nick-translated from the 6.7 kbp *Hin*dIII insert of p123/58-10. Plasmid DNAs isolated from strains which bound the probe were characterized by restriction enzyme analysis. A strain chosen for further work harbored pBt73-161, which contains the 3′-end of a crystal protein gene.

11.2 Construction of a full-length insecticide gene. The 5′- and 3′-ends of the protoxin genes were fused together at the unique *Hin*dIII site to form a complete protoxin gene. p123/58-10 DNA was digested with *Bam*HI, ligated to itself, and transformed into HB101. Plasmid DNAs from ampicillin-resistant transformants were characterized by restriction enzyme analysis, and a strain was identified that harbored a plasmid, designated pBt73-10(Bam), having single *Bam*HI and *Hin*dIII sites due to deletion of a small *Hin*dIII site-bearing *Bam*HI fragment. A 5 kbp *Hin*dIII fragment of pBt73-161, isolated by agarose gel electrophoresis, was mixed with and ligated to *Hin*dIII-digested dephosphorylated (by bacterial alkaline phosphatase) pBt73-10(Bam) DNA. After the ligation mixture was transformed into HB101, plasmid DNA isolated from ampicillin-resistant tetracycline-sensitive transformants was characterized by restriction enzyme analysis. A transformant was identified that harbored a plasmid, designated pBt73-16, carrying a complete protoxin gene. *E. coli* HB101 (pBt73-16) is on deposit at the Northern Regional Research Center, 1815 N. University Street, Peoria, Illinois 61604 USA, as NRRL B-15759.

Example 12

This Example teaches the insertion of the full-length *Bacillus thuringiensis* insecticide gene between a T-DNA gene promoter and a polyadenylation (poly(A) addition) signal, the transfer of the insecticide gene to tobacco *via* a sub-Ti plasmid, the regeneration of plants containing the gene, and the expression of this gene under control of the T-DNA promoter in plant cells.

12.1 Preparation of pH400, a sub-Ti plasmid. pH4-1 DNA can be isolated from *E. coli* K802 (pH4-1), which has been deposited as NRRL B-18009. pH4-1 is a sub-Ti plasmid based on pSUP106 (Preifer, U.B. *et al.* [1985] *J. Bacteriol.* 163:324-330; *E. coli* CSH52 (pSUP106) is deposited as NRRL B-15486). Inserted into pSUP106's *Hin*dIII site is pTi15955 DNA having border A (the left border of T_L), a plant-expressible selectable marker, the 5'-end of *tml*, a complete *ocs* gene, and border B (the right border of T_L). The plant-expressible marker is sutured to the adjoining pTi15955 sequences at *Bgl*II sites made up of a Tn5 neomycin phosphotransferase II (NPT2) structural gene capable of detoxifying kanamycin and related antibiotics (a *kan* gene). The NPT2 structural gene is transcribed in plant cells under control of a cauliflower mosaic virus (CaMV; for a review of CaMV see Hohn, T. *et al.*, *Curr. Top. Microbiol. Immunol.* 96:193-236) 19S transcript promoter and a pTi15955 ORF26 (as defined by Barker, R.F. *et al.* [1983] *Plant Mol. Biol.* 2:335-350) polyadenylation site. The NPT2 structural gene is oriented so that it is transcribed in the same direction as the *ocs* gene. In other words, the T-DNA of pH4-1 is essentially identical to T_L-DNA of pTi15955 except for substitution of a CaMV 19S promoter NPT2 structural gene/ORF26 polyadenylation site combination for T-DNA between the position 1617 *Bgl*II site and the position 11207 *Sma*I site, T-DNA positions being defined by Barker *et al.*, *supra*.

pH4-1 has two *Bgl*II sites, both of which flank the *kan* selectable marker. One of the *Bgl*II sites was removed, thereby leaving a unique *Bgl*II site useful for insertion of extraneous coding DNA. pH4-1 DNA was linearized by being partially digested with *Bgl*II and full-length, linear DNA was electrophoretically isolated. The *Bgl*II sticky-ends were then removed by incubation with the Klenow fragment of *E. coli* DNA polymerase I. The resulting blunt-ended DNA was ligated to itself and transformed into *E. coli*. Plasmid DNAs isolated from transformants resistant to chloramphenicol were screened by restriction analysis and a colony was identified which harbored a plasmid designated pH400. pH400 is identical to pH4-1 except for the absence of the *Bgl*II site between the *kan* gene and the ORF1 promoter, the

unique pH400 *Bgl*II site being located between the *kan* gene and the *ocs* gene. The single *Bgl*II site is a convenient place to insert DNA, in particular if that DNA has compatible 5'GATC . . . 3' sticky-ends resulting from the action of *Bgl*II, *Bcl*I, or *Bam*HI.

12.2 Preparation of pDOB513. pDOB512, carrying cauliflower mosaic virus (CaMV) transcription controlling sequences (obtained from Dr. Ken Richards, Centre National de la Recherche Scientifique, Institute de Biologie Moleculaire et Cellulaire, 15, rue Descartes, F-67084 Strasbourg, France) was constructed as follows: A *Hin*dIII fragment carrying the CaMV 19S RNA promoter region (CaMV nucleotides 5376-5851) was inserted into pBR322 and was trimmed back to within one base pair of the 19S transcript cap site. An adapter molecule having both a *Sma*I site and a *Bam*HI site (the structure being 5'-CCCGGGGATC CGG-3':5'-CCGGATCCCC GGG-3', see below) was then ligated to the trimmed DNA. A *Hin*cII fragment carrying the CaMV 19S transcript terminator (CaMV nucleotides 7018-7794) to which *Bam*HI linkers had been added was then inserted behind the 19S promoter, the promoter and terminator being separated by the *Sma*I/*Bam*HI linker. The resulting plasmid is designated pDOB412. pDOB412 DNA was digested with *Bgl*II and *Sal*I, filled in by incubation with the Klenow fragment of *E. coli* DNA polymerase I, and religated, thereby deleting DNA, which includes *Bam*HI and *Hin*dIII sites, between the CaMV position 7644 *Bgl*II site and the pBR322 position 650 *Sal*I site and regenerating a *Bgl*II site. The resultant plasmid was designated pDOB512.

The sticky-ends of *Hin*dIII-linearized pDOB512 DNA were converted to blunt-ends. The blunt-ended pDOB512 DNA was mixed with and ligated to commercially available *Bgl*II linkers. The ligation mix was transformed into *E. coli* K802 and an ampicillin-resistant transformant was isolated which harbored a plasmid, designated pDOB513 (Figure 3). pDOB513 has CaMV 19S transcription controlling sequences on a *Bgl*II fragment. *Sma*I and *Bam*HI sites are found between the DNA segments having the promoter and the polyadenylation site in both pDOB412, pDOB512, and

pDOB513, thereby providing a convenient location for insertion of foreign DNA that is to be a template for a transcript. The sequence of this suture is as follows:

```
                          BamHI
                    SmaI
5'...GAGAAAATCAGCCCGGGGATCCGGAACA...3'
```

The first underlined base, an A, corresponds to the first base of the 19S mRNA, transcription being towards the right, and the second and third underlined bases, respectively a G and an A, are derived from CaMV sequences at positions 5765 and 7018, respectively, as defined by Hohn *et al.*, *supra*.

12.3 Preparation of T-DNA ORF24 transcription controlling sequences. pKS-*pro*I was prepared essentially as described in Example 2.2. pSK111 is a recombinant plasmid having Ti15955 T-DNA which spans the sequence between *Eco*RI sites at positions 16,202 and 21,631 (as defined by Barker *et al., supra*) inserted into the *Eco*RI site of pRK290 (Ditta, G. *et al.* [1980] *Proc. Natl. Acad. Sci. USA* 77:7347-7351). *E. coli* K802 (Wood, W.B. [1966] *J. Mol. Biol.* 16:118) methylates DNA at many, but not all, *Cla*I sites, thereby specifically protecting certain sites from the action of *Cla*I. The only susceptible *Cla*I sites of pKS111 are the sites at T-DNA positions 18,892 and 20,128. These sites define a DNA fragment which includes the ORF24 (as defined by Barker *et al., supra*) structural gene but does not include the ORF24 promoter or polyadenylation site. ORF24 is associated with mannopine synthesis. Plasmid DNA isolated from *E. coli* K802 (pKS111) was digested with *Cla*I, ligated to itself, and transformed into K802. A tetracycline-resistant transformant was identified by restriction analysis which harbored a plasmid, designated pKS-*pro*I (or alternatively, pTR-*pro*I), identical to pKS111 except for deletion of a fragment which spanned the *Cla*I sites at positions 18,892 and 20,128.

pTR-*pro*I DNA was isolated and cleaved at its single *Cla*I site (the suture between the sites at positions 18,892 and 20,128) by incubation with *Cla*I. After the *Cla*I sticky-ends were then removed by incubation with the Klenow fragment of *E. coli* DNA polymerase I, the blunt-ended DNA was mixed with and ligated to

commercially available *Bam*HI linkers having the self-complementary sequence 5'-CGGATCCG-3'. After digestion of the *Bam*HI-linked DNA with *Bam*HI, religation to itself, and transformation of the resulting DNA into K802, plasmid DNAs were isolated from transformants resistant to tetracycline and were characterized by restriction mapping. A colony was identified which harbored a plasmid, designated pTR-*pro*I(Bam), lacking a *Cla*I site at the T-DNA positions 18,892/20,128 suture but, instead, having a *Bam*HI site at the former location of that *Cla*I site.

pBR322 has a *Bam*HI site which is inconvenient for later manipulations. Therefore, the T-DNA of the *Cla*I-deleted pKS111-derivative was transferred to a pBR325 derivative which was lacking a *Bam*HI site. pBR325 DNA, isolated from *E. coli* GM33 (pBR325), was digested with both *Bam*HI and *Bcl*I, was ligated to itself, and was transformed into *E. coli*. GM33 (Marinus, M.G., R.N. Morris [1974] *J. Mol. Biol.* 85:309-322) does not methylate adenine residues of DNA. Plasmid DNA isolated from transformants sensitive to tetracycline and resistant to chloramphenicol and ampicillin were characterized by restriction mapping, and a colony was identified which harbored a plasmid, designated pBR325aBB, which could not be cleaved with either *Bam*HI or *Bcl*I. pTR-*pro*I DNA was digested with *Eco*RI and was mixed with and ligated to *Eco*RI-linearized, dephosphorylated pBR325aBB DNA. After transformation into *E. coli*, plasmid DNAs isolated from transformants resistant to ampicillin and sensitive to both chloramphenicol and tetracycline were characterized by restriction mapping. A colony was identified which harbored a plasmid, designated p403B, having the *Cla*I-deleted T-DNA with *Bam*HI linkers in the former *Cla*I site, recombined with the pBR325aBB vector.

pKS4.2 DNA (Example 2.3) was digested with *Cla*I, and a fragment carrying the *kan* gene was electrophoretically isolated. This fragment was mixed with and ligated to *Cla*I-linearized pBR322 and transformed into *E. coli*. Plasmid DNAs isolated from transformants resistant to ampicillin and kanamycin were screened by restriction analysis and a colony was identified which harbored a plasmid designated pKS4.3. The pKS4.3 *kan* gene was oriented with its 5'-end and 3'-end respectively

proximal to the pBR322 *Eco*RI and *Bam*HI sites. In this orientation, the *kan* gene may be removed on a *Hin*dIII fragment.

*Bam*HI-linearized pBR322 DNA was mixed with and ligated to *Bgl*II-digested bacteriophage *lambda* DNA. Transformation into *E. coli* cells was followed by selection for resistance to ampicillin and screening for sensitivity to tetracycline. Plasmid DNAs were then isolated and screened by restriction analysis and a colony was identified which harbored a plasmid, designated pBR322Bam$^-$, which could not be cleaved with *Bam*HI.

pKS4.3 DNA was digested with *Hin*dIII and the *kan* gene-bearing fragment was electrophoretically isolated. The *kan* fragment was then mixed with and ligated to *Hin*dIII-linearized pBR322Bam$^-$DNA. The ligation mixture was then transformed into *E. coli*. Plasmid DNAs isolated from transformants resistant to both kanamycin and ampicillin were screened by restriction analysis and a colony was identified which harbored a plasmid, designated p11-83c, having the *kan* fragment inserted into pBR322Bam$^-$'s *Hin*dIII site. In p11-83c the *kan* gene was oriented so that the *kan* gene was transcribed in the same direction as the ampicillin resistance (*amp*) gene.

pTR-*pro*I(Bam) was digested with *Eco*RV and an approximately 2.26 kbp fragment bearing the ORF24 ("1.6" transcript) promoter and polyadenylation site, but lacking the ORF24 structural gene, was electrophoretically isolated. The *Eco*RV sticky-ends were then converted to blunt-ends by the action of the Klenow fragment of *E. coli* DNA polymerase I. The blunt-ended fragment was then mixed with and ligated to commercially available *Bgl*II linkers. After trimming off excess linkers by digestion with *Bgl*II, the fragment was mixed with and ligated to *Bgl*II-linearized p11-83c DNA. The ligation mixture was then transformed into *E. coli*. Plasmid DNAs isolated from ampicillin-resistant, kanamycin-sensitive transformants were restriction mapped and a colony was identified which harbored a plasmid, designated pCG116, having a *Bgl*II fragment bearing the ORF24 promoter and polyadenylation site inserted into Tn*5* DNA between the *kan* promoter and *kan* structural gene.

pDOB513 DNA was digested with *Bgl*II, religated to itself, and transformed into K802. Colonies which harbored a plasmid, designated pDOB514, deleted for CaMV transcription controlling sequences were identified by restriction mapping of the harbored plasmids.

pCG116 DNA, which had, on a 2.26 kbp *Bgl*II fragment, a *Bam*HI site between the ORF24 promoter and transcript terminator, was then digested with *Bgl*II, which cleaves at sites which in T-DNA correspond to positions 18,027 and 21,522 *Eco*RI sites. The restriction digested DNA was mixed with and ligated to *Bgl*II-linearized pDOB514 DNA. Plasmid DNAs of ampicillin-resistant transformants were characterized by restriction mapping, and a colony was identified which harbored a plasmid, designated pMAN514, having a 2.29 kbp *Bgl*II fragment carrying an ORF24 promoter and polyadenylation site separated by a *Bam*HI site.

12.4 Preparation of the insecticide gene. After pBt73-16 was digested with *Nde*I, resulting *Nde*I sticky-ends were filled in by incubating the mixture with T4 DNA polymerase and all four dNTPs. The blunt-ended *Bacillus* DNA was mixed with and ligated to double-stranded, *Sma*I-linearized, M13mp19 RF DNA (Norrander, J. *et al.* [1983] *Gene* 26:101-106). The ligation mixture was transformed into *E. coli* JM105. DNAs isolated from plaques that were "clear" when plated on indicator plates were characterized by restriction analysis and a plaque was identified which harbored a vector, designated 1.6.4, having a 3.6 kbp *Bacillus thuringiensis* DNA oriented such that single-stranded form was complementary to crystal protein mRNA (i.e., the phage carried the antisense strand).

A *Bam*HI site was introduced into the *Bacillus* DNA immediately 5′ to the crystal protein translational start site essentially as described in Example 10. Sequences of the unmutated *Bacillus* DNA and the oligonucleotide primer are as follows:

```
                                  TAA
Bacillus:  5'...GAGATGGAGG    CTTATGGATAAC...3'
Primer:       5'GAGATGGAGGATCCTTATGGATAAC3'
                           BamHI   MetTyrHis
```

Putative mutant DNAs were screened for hybridization under stringent conditions to a ^{32}P-label derivative of the oligonucleotide primer and for the presence of a novel *Bam*HI site. A plaque was identified harboring a vector designated 1.6.4B-3.8.3, having a *Bam*HI site immediately 5′ to the translational start. Fidelity of the *in vitro* mutation was confirmed by DNA sequencing. The insecticidal protein structural gene may be removed from double-stranded 1.6.4B-3.8.3 RF DNA on a 3.75 kbp *Bam*HI fragment resulting from cleavage at the novel site in the *Bacillus* sequence and a *Bam*HI site which is 3′ to the structural gene in the M13mp19 polylinker.

12.5 Combination of an insecticidal protein structural gene with ORF24 transcription controlling sequences. Double-stranded 1.6.4B-3.8.3 RF DNA and pCG116 DNA, both having been digested with *Bam*HI, were mixed with each other and ligated together. After the ligation mixture was transformed into *E. coli*, plasmid DNAs isolated from ampicillin-resistant transformants were characterized by restriction analysis. A colony was identified which harbored a plasmid, designated pCJ161, having a full-length insecticidal protein structural gene placed between the ORF24 promoter and ORF24 polyadenylation site in an orientation such that the mRNA transcribed under control of the ORF24 promoter encodes the insecticidal protein.

12.6 Insertion of a plant-expressible insecticide gene into a sub-Ti plasmid. pCJ161 and pH400 DNAs, having been digested with *Bgl*II, were mixed with each other and ligated together. After the ligation mixture was transformed into *E. coli*, plasmid DNAs isolated from ampicillin-resistant transformants were characterized by restriction analysis. A colony was identified which harbored a plasmid, designated pH450, having the ORF24 promoter/crystal protein structural gene/ORF24 polyadenylation site combination inserted into the pH400 *Bgl*II site, which is located between the *ocs* gene and the plant-expressible *kan* gene. The plant-expressible crystal protein gene of pH450 is oriented so that it is transcribed in the same direction, i.e., off of the same DNA strand, as both the *ocs* gene and the *kan* gene.

12.7 Plant transformation. pH450 was transferred into *A. tumefaciens* LBA4404 (Ooms, G., *et al.* [1981] *Gene* 14:33-50), a *vir* gene-bearing micro-Ti-mobilizing strain, by the triparental mating technique. Tobacco leaf tissues were inoculated using a modification of the method of Horsch, R.B. *et al.* (1985) *Science* 227:1229-1231. Leaf segments were dipped in the inoculating suspension of bacteria for 1-3 minutes. The inoculating bacterial suspensions had titres of 10^7-10^8 ml^{-1}; the exact concentration was found to be unimportant. The medium was a tobacco regenerating medium having MS salts, a mixture well known to the art, supplemented with 0.1 mg/l p-chlorophenoxyacetic acid (pCPA), 7.5 mg/l 6-(2,2-dimethylallylamino)-purine (2iP), 300 mg/l kanamycin, and 125 mg/l cloxacillin, mefoxin and 500 mg/l carbenicillin. Generally 75-80% of the shoots were able to root in a kanamycin-containing medium and all were transformed. Some kanamycin-resistant shoots did not make measurable quantities of octopine.

12.8 Expression in plant tissues: immunoassays. Micro-ELISA double antibody sandwich assays were performed using a modification of the method of Example 7.

Antibodies were prepared by well-known methods. Coating antibody was partially purified mouse polyclonal antisera which was raised to *B. thuringiensis* HD-73 crystal protein. The mouse antisera was partially purified by precipitation with 50% (w/v) saturated ammonium sulfate at pH 7.4. The milky precipitate that forms during 30 minutes on ice was pelleted by centrifugation (20 minutes, 10,000 rpm). The pellet was then resuspended in phosphate buffered saline (PBS, pH 7.4) containing 137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 8 mM $NaHPO_4$. The resulting coating antibody sample was then dialyzed overnight in PBS at 4°C.

Primary antibody was partially purified rabbit polyclonal antisera raised to *B. thuringiensis* HD-73 crystal protein. This antisera was cleared of antibodies that react with tobacco leaf protein. Tobacco leaf protein was bound to CNBr-activated "SEPHAROSE 4B" (Pharmacia, Upsalla, Sweden) (10 mg protein/ml gel). Rabbit serum was diluted 1:4 (v/v) with PBS and added to the swollen "SEPHAROSE" gel

to which the leaf protein was bound. About 2-3 ml of gel was used for 4 ml of diluted serum. The serum-gel mixture was incubated with gentle shaking for about 1 hour at room temperature. Antibodies that react with leaf protein remain bound to the gel. The liquid containing unbound antibodies was removed from the gel matrix by aspiration. The antibody solution was then partially purified by precipitation with 50% saturated ammonium sulfate followed by overnight dialysis against PBS at 4°C.

Secondary antibody was commercial goat anti-rabbit IgG (H+L) labeled with alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD). Sterile, distilled water (1 ml) was added to 0.5 mg of lyophilized pellet. The wells of disposable flexible 96-well/flat bottom microtitration plates (Dynatech, Alexandria, VA) were coated (100 μl/well) with coating antibody (1:2000 dilution of coating antibody in coating buffer). Coating buffer contained 5 mM Na_2HCO_3 and 35 mM $NaHCO_3$ at pH 9.6 After addition of the coating antibody solution, the plates were covered and stored at 4°C for at least 12 hours. The contents of the wells were then discarded and the entire plate was rinsed about 3-4 times with a wash buffer of PBS containing 0.05% polyoxyethylenesorbitan monolaurate ("TWEEN-20"). Plates were then filled with wash buffer and placed on a shaker for 5 minutes followed by a final rinse. The plates were then drained for 30 seconds. It is important that at no point in the assay the wells dry for a prolonged period. In between assay steps the wells were filled with wash buffer.

The plates were next treated with blocking solution to prevent non-specific binding of proteins. Plate wells were filled with blocking solution containing 1% (w/v) bovine serum albumin (BSA); 1% (w/v) casein, acid hydrolysate, type 1; and 0.05% (v/v) polyoxyethylenesorbitan monolaurate ("TWEEN-20") and allowed to stand at room temperature for 30 minutes to 1 hour.

Tobacco plant tissue samples were prepared from leaf material. Generally, 1 or 2 leaves from the apex of the plant were combined with 4 or 5 leaf discs (7 mm) from the basal and marginal regions of the next youngest leaves. Tissue samples of

about 100 mg were used. After weighing, tissue samples were quick-frozen in liquid nitrogen and ground in a tissue grinder. The sample was allowed to warm to room temperature during grinding. After the tissue was completely ground, PBS was added to make a solution containing 0.3 mg fresh weight/μl (for a 100 mg sample, 500 μl of PBS was added). Callus tissue samples were prepared in the same manner, except that PBS was added to ground tissue to give a solution of 0.5 mg fresh weight/μl. Samples were then mixed thoroughly and allowed to stand at 4°C (about 20 minutes) for tissue to settle out. Samples (10-15 μl) of the supernatant were taken for total protein assays. Samples were then diluted with an equal volume of a 2x concentration of sample buffer. Sample buffer (1x) contained PBS (pH 7.4) with 2% (v/v) polyethylene glycol, 1% (v/v) polyvinyl pyrolidone, 0.1% (w/v) BSA, 0.1% (w/v) casein, acid hydrolysate, 10 μg/ml "APROTININ" (Sigma), a protease inhibitor, 0.05% (v/v) polyoxyethylenesorbitan monolaurate, and 0.006% (v/v) β-mercaptoethanol. Control tissue (non-transformed) samples were prepared in a similar manner. Samples were spun in a microfuge for 1 minute before loading into wells of the prepared microtiter plates. Sample supernatant was loaded (100 μl/well) in replicates (3 or 4). Plates containing samples are incubated for 2-3 hours at room temperature. For quantitation, standards of *B. thuringiensis* crystal protein can be included.

After incubation the contents of the wells were removed and the plate was rinsed carefully with wash buffer (4-5 times). The plate was filled with wash buffer and agitated for 5 minutes. Care should be taken in this step to avoid cross-contamination of samples, controls, and standards.

A 1:2000 dilution of the primary antibody in antibody buffer was then loaded into the wells (100 μl/well). Plates were covered and stored at 4°C overnight (about 12 hours). Antibody buffer contained PBS (pH 7.4) with 1% (v/v) goat serum and 0.05% polyoxyethylenesorbitan monolaurate. Plates were then washed (3-4 times) with wash buffer (as above). Plates were then blotted dry.

A 1:2500 dilution of labeled secondary antibody (goat anti-rabbit IgG (H+L)-alkaline phosphatase-labeled) was then loaded into wells (100 μl/well). Plates were incubated for 1-2 hours at room temperature and thoroughly washed. Plates were washed 4-5 times with wash buffer. Plates were filled with wash buffer and placed on a shaker for at least 10 minutes. Plates were rinsed again and blotted dry.

An NADH substrate was then employed to detect bound labeled antibody. NADP (β-nicotinamide adenine dinucleotide phosphate) 0.25 mg/ml in 0.50 mM $MgCl_2$, 0.05 mM diethanolamine (pH 9.5) was loaded into the assay wells (100 μl/well). Plates were covered and stored at 4°C for about 1-1/2 hours.

Reagent solutions was prepared by dissolving p-Iodonitrotetrazolium violet (0.31 mg/ml) in 0.05 M sodium phosphate buffer (pH 7.2) containing 1.9% (v/v) ethanol. To this solution "DIAPHORASE" (NADH/dye oxidoreductase; Boehringer Mannheim, Mannheim, West Germany) to 0.035 mg/ml and alcohol dehydrogenase (baker's yeast) to 0.035 mg/ml were added. The reagent solution was incubated at 4°C for 45 minutes, after which it was added to the NADP solution in each assay well (150 μl/well).

The color reaction (red) should begin immediately. Optical density measurements were read at 492 nm.

Adjusted ELISA values of crystal protein in leaf tissue, expressed as absorbance at 492 nm, are reported in Table 7. The values were adjusted by subtracting from each measurement the value obtained for a *Nicotiana tabacum* var. "Xanthi" control. Many plants were observed to contain measurable amounts of antigen which bound anti-crystal protein antibody.

12.9 Expression in plant tissues: bioassays. Tobacco tissue transformed by pH450 was cloned and maintained in a growth chamber. For bioassay, leaves were cut off with a razor blade and placed in a 9 cm Petri dish lined with moist filter paper. Newly-hatched tobacco hornworm larvae (*Manduca sexta*) were put on the leaves. Mortality was recorded.

In trial 1, three clones from each type were sampled with five larvae per dish (Table 8). The data can be summarized as follows:

Clone	Total	Dead	% Dead
100	14	5	36
103	15	1	7
106	15	0	0
109	15	5	33
111	15	3	20

Trial 2 was essentially done as described for trial 1, but here two larvae were placed in each dish. The data are reported in Table 9 and can be summarized as follows:

Clone	Total	Dead	% Dead
100	14	9	64
103	18	4	22
106	24	12	50
109	26	7	27
111	18	8	44

Leaves that had killed both larvae by day 2 were then reinfested with three newly hatched larvae. While the numbers were not large enough to make comparisons between treatments, it was clear that these leaves are still toxic. For example, three dishes from clone 100 received a total of 9 larvae, 6 of which were dead 2 days later.

As protein concentration is highest in small leaves, expression might vary with the size of the leaf, i.e., with the degree of expansion of the cells. Therefore in trial 3 leaves were measured before bioassay. For this test, only plants that had not been sampled before of clones 100 and 103 were sampled. The data were ranked from smallest to largest leaves, but the sizes for the two clones were not aligned. Different

numbers of dishes were set up for each plant because the plant of clone 100 had fewer leaves. Three larvae were placed in each dish. Number of dead larvae per dish are reported below.

Rank	Clone 100	Clone 103
1	1	0
2	3	0
3	1	0
4	1	0
5	1	0
6	3	0
7	3	0
8		0
9		2
10		1

It is clear that both small and large leaves are able to kill the larvae. Furthermore, the difference between different plant clones, i.e., 100 and 103, is apparent: 62% vs. 10% mortality, at day 2.

In trial 4, randomly assigned labels were used to avoid biases. Five Petri dishes from each clone were prepared with three larvae per dish. In addition, a Xanthi plant that originated in tissue culture was used as a control. The data can be summarized as follows:

Summary	Dead/Total	% Mortality
control	2/15	13
100	11/15	73
103	8/15	53
106	2/15	13
109	1/15	7
111	3/15	20

Clone 100 gave consistently high mortality. Clone 103 also gave high mortality explained by the subsequently discovered fact that "clone" 103 was not in fact a clone; the original transformed plant has now been shown to have been a chimeric plant. It has not proven to be particularly insecticidal in any other of the five trials.

After the trial 4 leaves had been fed on for two days, ELISAs were performed on some of the lethal and nonlethal leaves. No crystal protein antigen was detected because of the low levels of protein in these leaves.

In trial 5, four plants were propagated from clone 100 and five from clone 103. Five leaves were sampled from each of these plants. Each leaf was placed in an individual Petri dish with three newly hatched larvae. The data can be summarized as follows:

Summary	Dead/Total	% Mortality
100	8/14	57
100	3/14	21
100	10/15	67
100	5/14	36
103	3/15	20
103	1/15	7
103	2/15	13
103	2/15	13

Thus, clone 100 is consistently more toxic than clone 103.

Southern blot analysis of DNA isolated from presumptive transformants showed that cloned plant tissue having insecticidal activity in bioassays and containing crystal protein antigen generally had pH450's T-DNA. Northern blot analysis of RNA generally demonstrated the presence of mRNA having crystal protein sequences. These mRNA molecules were not the expected size of about 3.8 kbp, but were about 1.7 kbp in size. This was sufficient to encode the toxic portion of the crystal protein. As predicted from the fact that crystal protein levels in leaves as

estimated by ELISA were below the limit of detection on Western blots, Western blot analysis of proteins extracted from transformed tissues did not reveal any antigens that cross-reacted with anti-crystal protein antibodies. Polypeptides that bind anti-crystal protein antibodies have been detected by Western blot analysis of both transformed callus and young shoot tissues.

Example 13

This Example teaches more DNA constructions useful for transformation of a number of species of plants.

13.1 Plant transformation vectors. pH450 was described in Example 12.6. pH575 was disclosed by Sutton *et al.* (1987) European Patent Publication 0 223 417 (priority document: U.S. Patent Application Serial No. 06/788,984, filed October 21, 1985, which is hereby incorporated by reference). pH576, pH577, pH578, pH582, and pH585 are derivatives of pH575, having various promoter/insecticide structural gene/polyadenylation site combinations, all oriented parallel to the *ocs* gene and the plant-selectable *kan* gene and inserted into the *Bgl*II site of pH575 between those two genes.

13.2 Modification of an insecticide gene's 5'-end. Construction, isolation, and characterization of pNSBP544 were disclosed by Sekar *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:7036-7040; and Sekar and Adang, U.S. Patent Application Serial No. 07/108,285, which is hereby incorporated by reference. A 3.0 kbp *Hin*dIII fragment carrying the crystal protein gene of pNSBP544 was inserted into the *Hin*dIII site of pIC-20H (Marsh *et al.* [1984] *Gene* 32:481-485), thereby yielding a plasmid designated p544-*Hin*dIII, which is on deposit. 73 kDa crystal protein may be expressed in *E. coli*. The 73 kDa species is processed to form the 65 kDa species by removal of 49 amino acids at the amino-terminus of the 73 kDa species, leaving alanine at the amino-terminus of the 65 kDa species.

A 5.9 kbp *Bam*HI fragment carrying the crystal protein gene was removed from pNSBP544 and inserted into *Bam*HI-linearized pIC-20H DNA. The resulting

plasmid, p405/44-7, was digested with *Bgl*II and religated, thereby removing *Bacillus* sequences flanking the 3'-end of the crystal protein gene. The resulting plasmid, p405/54-12, was digested with *Pst*I and religated, thereby removing *Bacillus* sequences flanking the 5'-end of the crystal protein and about 150 bp from the 5'-end of the crystal protein structural gene. The resulting plasmid, p405/81-4, was digested with *Sph*I and *Pst*I and was mixed with and ligated to a synthetic linker having the following structure:

```
          SD        MetThrAla
 5'CAGGATCCAACAATGACTGCA3'
3'GTACGTCCTAGGTTGTTACTG5'
  SphI                  PstI
```

(SD indicates the location of a Shine-Dalgarno prokaryotic ribosome binding site.) The resulting plasmid, p544Pst-Met5, contains a structural gene encoding a protein identical to one encoded by pNSBP544 except for a deletion of the amino-terminal 47 amino acid residues. The protein encoded by p544Pst-Met5 is 2 amino acids longer than the 65 kDa toxic polypeptide processed from the 75 kDa crystal protein encoded by pNSBP544 (data not shown; see also McPherson *et al.* [1988] *Biotechnol.* 6:61-66). In bioassays, the proteins encoded by pNSBP544 and p544Pst-Met5 were shown to be equally toxic. All of the plasmids mentioned above have their crystal protein genes in the same orientation as the *lacZ* gene of the vector.

13.3 Modification of an insecticide gene's 3'-end. A *Hin*dIII site and a *Xma*I site were removed from the kanamycin resistance gene (*kan*), which encodes neomycin phosphotransferase I (NPTI), of pUC4K (Viera and Messing [1982] *Gene* 19:259-268) by the method of Merlo and Thompson (1987) *Anal. Biochem.* 163:79-87. The *kan* gene was removed from the resulting plasmid on a *Hin*cII fragment, and the ends were filled in by T4 DNA polymerase to make sure that they were blunt. pIC-20R (Marsh *et al.*, *supra*) DNA was digested with *Nde*I and *Sca*I and the ends were filled in by T4 polymerase. The resulting DNA, lacking the 5'-end of the ampicillin-resistance gene (*amp*) was mixed with and ligated to the NPT1-encoding *Hin*cII

fragment. A plasmid having *kan* in the same orientation as *amp* was identified and labeled pIC-20RXmn$^-$Kan^r.

A *Hin*dIII fragment carrying the crystal protein gene was removed from p544Pst-Met5 and inserted into the *Hin*dIII site of pIC-20RXmn$^-$Kan^r. A plasmid having the crystal protein gene oriented antiparallel to the pIC-20R *lac*Z gene was identified and designated p461/80-159. p461/80-159 DNA was digested with *Bgl*II and *Xmn*I which opened it at the *Bgl*II site of the pIC-20R polylinker and just after nucleotide 1778 (*Xmn*I) of the crystal protein gene. The opened plasmid was mixed with and ligated to a synthetic DNA linker having the following structure:

```
     PheIleProValAsnLeuArgSer
5'TATCCCAGTGAATTTAA3'
   3'ATAGGGTCACTTAAATTCTAG5'
                        BglII
```

(The underlined base pair indicates a mutation that eliminates the *Xmn*I site present in the wild-type gene.) The resulting plasmid was designated p461/97-14.

13.4 Fusion of an insecticide gene with NPT2 sequences. The *kan* gene of Tn5 was modified as follows (see Merlo *et al.*, European Patent Application Publication No. 0 233 417, priority U.S. Patent Application Serial No. 06/788,984, continued by Serial No. 07/144,016). Coordinates refer to the sequences published by Beck *et al.* (1982) *Gene* 19:327-336. The G at position 144 was changed to a C, thereby introducing a *Bam*HI site. The C at position 148 was changed to an A, thereby improving the efficiency of eukaryotic translational initiation at the AUG at position 151 to 153. The *Sma*I site that cuts after position 1118 was changed to a *Bgl*II site by digestion with *Sma*I followed by ligation to *Bgl*II linkers. The NPT2 gene is carried by a 0.98 kbp DNA fragment after digestion with *Bam*HI and *Bgl*II.

The 0.98 kbp fragment was mixed with and ligated to *Bgl*II-digested p461-97-14 (Example 13.3). A plasmid, p461/151-174, was identified having the NPT2 sequence oriented with its 5'-end *Bam*HI site fused with the *Bgl*II site proximal to the truncated Btt crystal protein gene of p461/97-14. The suture of p461/151-174 between the Tn5

and *B. thuringiensis* sequences was sequenced to confirm conservation of reading frame.

13.5 Construction of transcription vectors. An 833 base pair (bp) fragment was cut out of the pUC13 clone carrying the "−343" deletion (Odell *et al.* [1985] *Nature* 313:810-812) by digestion with *Sma*I and *Hin*dIII. This fragment carries a functional 35S promoter and the 5'-end of the CaMV 35S transcript, spanning from position −343 to position +9 relative to the transcriptional start site. This fragment was ligated into pIC19R (Marsh *et al.* [1984] *Gene* 32:481-485) which had been digested with *Nru*I and *Hin*dIII. The ORF25 polyadenylation site was contributed by a pTi15955 fragment spanning *Hin*cII sites at positions 21,727 and 22,440, as disclosed by Barker *et al.* (1983) *Plant Mol. Biol.* 2:335-350, ligated into the *Sma*I site of pIC19H (Marsh *et al.*, *supra*), the ORF25 polyadenylation site being proximal to the *Bam*HI site in the pIC19H polylinker. The T-DNA was then removed from the pIC19H vector on a *Bam*HI/*Bgl*II fragment which was then inserted into the *Bam*HI site of the pIC19R/35S promoter combination, the T-DNA being oriented so that the ORF25 polyadenylation site was proximal to the 35S promoter, a functional *Bam*HI site was between the CaMV and T-DNA sequences, and a *Bam*HI/*Bgl*II fusion was between the T-DNA and pIC19R sequences. This plasmid was then opened at the *Sma*I site between the fused *Bam*HI/*Bgl*II site and the pIC19R vector sequences. Plural *Bgl*II linkers were ligated into the *Sma*I site, resulting in formation of a *Pst*I site between the linkers. The resulting plasmid was designated pIC35/A.

A DNA linker encoding the tobacco mosaic virus (TMV) 5'-leader sequence was synthesized. The linker had the structure of

```
  (BglII)                              TthIIIII
5' GATCTATTTTTACAACAATTACCAACAACAACAAACAA...
3'     ATAAAAATGTTGTTAATGGTTGTTGTTGTTTGTT...

                                                  (BamHI)
...CAAAACAACATTACAATTACTATTTACAATTACG      3'
...GTTTTGTTGTAATGTTAATGATAAATGTTAATGCCTAG 5',
```

the horizontal lines indicating the location of sites recognized or partially recognized (partial recognition is indicated by parentheses) by the indicated restriction enzymes. This linker was inserted into pIC35/A's *Bam*HI site. The resulting plasmid, designated pIC35/A-TL4, had a *Bam*HI site between the TMV leader and the ORF25 polyadenylation site.

An NPT2 expression vector was constructed having the same CaMV DNA fragment carrying the 35S promoter as pIC35, the same NPT2 structural gene as used in Example 14.2.2 to construct p461/151-174, and the same T-DNA ORF25 polyadenylation site as used in pIC35/A. A 3.0 kbp *Hin*dIII fragment carrying the truncated Btt crystal protein gene of pNBSP544 was inserted into pIC19R-Kan or p35S-Kan.

13.6 Assembly of direct transformation vectors. The insecticide structural gene/fusion linker combination of p461/97-14 was removed by digestion with *Bgl*II and *Bam*HI and inserted into *Bam*HI-linearized pIC35/A-TL4 DNA. A plasmid, designated p461:151-193, was identified which had the insecticide structural gene oriented so that the 5'-end of the insecticide structural gene was proximal to the 35X promoter and the 3'-end of the linker was proximal to the ORF25 polyadenylation site.

The insecticide structural gene/fusion linker/NPT2 structural gene combination of p461/151-174 was removed by digestion with *Bgl*II and *Bam*HI and inserted into *Bam*HI-linearized pIC35/A-TL4 DNA. A plasmid, designated p461:162-191, was identified which had the insecticide structural gene oriented so that the 5'-end of the insecticide structural gene was proximal to the 35S promoter, and the 3'-end of the NPT2 structural gene was proximal to the ORF25 polyadenylation site.

13.7 Fusion of an insecticide gene with hygromycin sequences. An insecticide structural gene was derived from a 5.3-class gene (Kronstad and Whiteley [1986] *Gene* 43:29-40) from *B. thuringiensis* HD-1 (pBT1-106A, disclosed by Adang *et al.* [1987] in *Biotechnology in Invertebrate Pathology and Cell Culture* [K. Maramorosch,

ed.], pp. 85-99). The 3'-end of this gene was removed by digestion with *Bal*31 and a linker having the structure

```
5' TAATTGAATAGATCT 3'
   3' ATTAACTTATCTAGA
```

was ligated onto the end. This linker encoded stop codons in all three reading frames and a *Bgl*II site at its 3'-end. The resulting structural gene had 1820 bp from the 5'-end of the structural gene, encoding 607 amino acids and an extra asparagine residue, encoded by the linker at the carboxy terminus. The 3'-end of the structural gene, therefore, had the structure:

```
                IleAspArgIleGluPheValProAlaAsnEnd BglII
5' ...ATAGATCGAATTGAATTTGTTCCGGCTAATTGAATAGATCT...3'
```

where the underlined sequence at the left indicates the sequence which in other experiments was shown to be insufficient to be insecticidal.

A hygromycin gene (*hyg*) (Gritz and Davies [1983] *Gene* 25:179-188) was modified by a G residue at position −3 relative to the translation start codon, and by insertion of a *Bam*HI site 5' to the translational start codon. Having a G at −3 is though to enhance translational initiation in eukaryotes. This *hyg* gene was ligated to the *Bam*HI site of the truncated HD-1 ("DIPEL") gene, thereby forming a DNA sequence which can encode a dicistronic mRNA. The polypeptide encoded by the truncated "DIPEL" gene was shown to be as toxic as the protein encoded by the full-length "DIPEL" gene.

13.8 Description of binary vectors. pH576 was a pH575 derivative carrying an ORF24 promoter/insecticide structural gene/ORF24 polyadenylation site combination identical to that carried by pH450.

pH577 was a pH575 derivative carrying a CaMV 19S promoter/insecticide structural gene/CaMV polyadenylation site combination, the promoter and polyadenylation site being carried by the same fragments which contributed them to

the plant-selectable *kan* genes of pH450 and pH575. pH577 had the same *Bacillus thuringiensis* HD-73 insecticide structural gene as pH450.

pH578 was a pH575 derivative carrying a CaMV 35S promoter/insecticide structural gene/T-DNA ORF25 polyadenylation site combination. pH578 had the same *Bacillus thuringiensis* HD-73 insecticide structural gene as pH450 and the same promoter and polyadenylation site as carried by pIC35/A.

pH582 was a pH575 derivative carrying a CaMV 35S promoter/insecticide structural gene/T-DNA ORF25 polyadenylation site combination. The promoter and polyadenylation site were the same used in pH578. The insecticide structural gene was derived from a 5.3-class gene (Kronstad and Whiteley [1986] *Gene* 43:29-40) from *B. thuringiensis* HD-1 and included about the 1.83 kbp 5′ from the structural gene (pBT1-106A, disclosed by Adang *et al.* [1987] in *Biotechnology in Invertebrate Pathology and Cell Culture*, K. Maramorosch, ed., pp. 85-99). The portion utilized encodes essentially only the toxic region of the insecticidal protein.

pH585 was a pH575 derivative carrying an ORF24 promoter/insecticide structural gene/ORF24 polyadenylation site combination, the insecticide structural gene being the same as contained by PH582. The promoter and polyadenylation site were the same as used in pH450 and pH576.

pH591 was a pH575 derivative carrying an ORF24 promoter dicistronic gene/ORF24 polyadenylation site. The dicistronic gene was the truncated "DIPEL" structural gene/*hyg* structural gene combination described in Example 13.7.

pH610 was a binary vector based on a pTJS75 replicon (Klee *et al.* [1985] *Biotechnol.* 3:637-642), previously used herein to make pH450 and pH575. The tetracycline resistance gene (*tet*) of pTJS75 had been replaced with an erythromycin resistance gene (*ery*) derived from pR29 (Morrison *et al.* [1984] *J. Bacteriol.* 159:870-876). From "left" to "right," the T-DNA of pH610 consisted of a pTi15955 (number scheme as in Barker *et al.* [1983] *Plant Mol. Biol.* 2:334-350) fragment spanning positions 602 to 1186 and carrying the left T_L-DNA border repeat; a neomycin phosphotransferase I (NPT1) gene, expressed in bacteria, originally from Tn903

(excised on a 1.45 kbp *Bam*HI fragment from pUC4K, Viera and Messing [1982] *Gene* 19:259-268), oriented to transcribe from left to right; a plant-expressible hygromycin-resistance gene, transcribed from right to left, under control of the CaMV 35S promoter and the T-DNA ORF25 polyadenylation site from pIC35/A, the hygromycin-resistant gene being derived from pLG62 (Gritz and Davies [1983] *Gene* 25:179-188); a dicistronic plant-expressible gene transcribed from right to left under control of the ORF24 promoter and either the ORF25 or ORF26 polyadenylation site (Barker *et al.*, *supra*), the dicistronic gene having an insecticide structural gene from HD-1 ("DIPEL") (a 4.5 kb-class gene, Kronstad and Whiteley, *supra*) at its 5′-end and a NPT2 structural gene (*kan*) at its 3′ end; and a T-DNA fragment spanning positions 12,070 to 14,710, carrying an octopine synthase gene (*ocs*) transcribed to the left and the right T_L-DNA border repeat.

pH615 was identical to pH610 with some exceptions. There was no NPT1 gene. In the plant-expressible selectable marker, a Tn5 NPT2 structural gene (the "double mutant" of Sutton *et al.*, European Patent Publication No. 0 223 417) and an ORF26 polyadenylation site (from the same *Hin*cII fragment as contributed the ORF25 site) substituted from the hygromycin resistance structural gene and the ORF25 polyadenylation site of pH610. The plant-expressible insecticide gene was terminated by an ORF25 polyadenylation site. The insecticide structural gene was from HD-1 ("DIPEL"), and was preceded by a slightly modified alfalfa mosaic virus (AMV) RNA_4 5′-leader sequence. This leader was encoded by a synthetic linker having a structure of

```
(HindIII)                              HpaI          (NcoI)
5′ AGCTTGTTTTTATTTTTAATTTTCTTTCAGTTAACTTCCAC      3′
3′      ACAAAAATAAAAATTAAAAGAAAGTCAATTGAAGGTGGTAC 5′
```

the horizontal lines indicating the location of sites recognized or partially recognized (partial recognition is indicated by parentheses) by the indicated restriction enzymes. This linker could be ligated to the *Hin*dIII site at the 3′-end of the 35S promoter, the resulting transcripts having 9 bp of the CaMV 35S transcript as its 5′-end. The *Nco*I

sticky-end could be ligated to the 5′-end of a structural gene by use of appropriately-tailed linkers.

pH619 was essentially identical to pH615 except for the insecticide structural gene and the polyadenylation site 3′ therefrom. The insecticidal structural gene of pH615 was the same as that carried by p544Pst-Met5, but lacking almost totally *Bacillus* sequences 3′ from the translational stop codon. The polyadenylation site 3′ from the structural gene was from T-DNA ORF25 and was carried, as described elsewhere herein, on a *Hin*cII fragment. Additionally, a TMV 5′-leader sequence (Example 13.5) was present between the 35S promoter and the p544Pst-Met5 structural gene.

pH623 was essentially identical to pH610 except for the presence of the TMV5′-leader (Example 13.5) at the 5′-end of the structural gene and for the substitution of the insecticide structural gene carried by p461/97-14 for the insecticide structural gene of pH610. The 3′-extension of the coding sequence beyond the natural position of the translational termination site did not affect toxicity of the encoded insecticidal protein.

pH624 was essentially identical to pH619 except for substitution of the insecticide/NPT2 structural gene of p461/151-174 for the insecticde structural gene of pH619.

pH627 was identical to pH615 with the exception of the presence of an inserted phaseolin third intron in the AMV RNA_4 5′-leader sequence. A fragment of a phaseolin gene, carrying the third intron and flanking coding sequences, and spanning from the *Xba*I site at position 904 to the *Sau*3AI site at position 1061 (as numbered by Slightom *et al.*, *supra*). This fragment could be inserted into the *Hpa*I site of the AMV RNA_4 leader linker with the aid of appropriate linkers to adapt *Xba*I and *Sau*3A sticky-ends to the blunt ends of *Hpa*I.

13.8 Deposited strains. The following strains were deposited with the Patent Culture Collection, Northern Regional Research Center, 1815 N. University Street, Peoria, Illinois 61604:

Strain	Deposit Date	Accession #
E. coli MC1061 (p544-HindIII)	10/06/87	NRRL B-18257
E. coli MC1061 (p544Pst-Met5)	10/06/87	NRRL B-18258

The deposited strains are provided for the convenience of those in the art, and are not necessary to practice the present invention, which may be practiced with the present disclosure in combination with publicly available protocols, information, and materials. *E. coli* MC1061, a good host for plasmid transformations, was disclosed by Casadaban and Cohen (1980) *J. Mol. Biol.* 138:179-207.

Example 14

This Example teaches expression in *Zea mays* of a coleopteran-active insecticidal protein gene from *Bacillus thuringiensis* var. *tenebrionis*.

14.1 Maize protoplast transformation. A suspension culture of Black Mexican Sweet (BMS) corn cells was digested in 2% cellulase, 0.25% pectinase (both from Worthington Biochemical Corp., Freehold, NJ), 0.2 M mannitol, and 80 mM $CaCl_2$ for about 4 hours at a concentration of about 1 g fr. wt. cells in 10 ml enzyme solution. The protoplast solution was filtered through a 46 μ mesh sieve to remove undigested cell clumps. Protoplasts were obtained from 8.5 g fr. wt. of cells. After washing, the protoplasts were resuspended in MaMg (0.4 M mannitol, 15 mM $MgCl_2$, 0.1% MES, pH 5.6) at a concentration of 2.5 x 10^6 ml^{-1}. Half ml aliquots of protoplasts were placed into 15 ml disposable round-bottom centrifuge tubes. Appropriate DNA solutions were added to each tube, followed by the addition of 300 μl of a 40% (w/v) polyethylene glycol (PEG) solution (avg. PEG molecular weight = 6000-7500; source: EM Science, Gibbstown, NJ; dissolved in NaMg; final pH about 6.0; filter-sterilized). DNA solutions were as follows:

	BTT	Fusion-High	Fusion-Low	Control
μg p461:162-191 DNA	--	10	2.5	--
μg p461:151-193 DNA	25	--	--	--
μg pIC19R-35S-Kan DNA	2.5	--	--	--
μg salmon sperm carrier DNA	22.5	40	47.5	50
Total DNA conc. (μg/ml)	100	100	100	100

The tubes were incubated for 30 minutes at room temperature with occasional gentle mixing. The incubations were then diluted by addition of and mixing with 1 ml of MS4D+8M (MS salts, 4 mg/l 2,4-D, 8% mannitol, 2% sucrose, 0.75 mg/l thiamine·HCl, 7.7 mg/l glycine, 1.3 mg/l nicotinic acid, 0.25 mg/l pyridoxine·HCl, 0.25 mg/l calcium pantothenate, and 1 mM asparagine). After a further 5 minutes, two further dilutions of 2 ml MS4D+8M were done at 5 minute intervals. The protoplasts were centrifuged at low speed, resuspended at a concentration of 2×10^5 m^{-1} in CM + 8% mannitol (CM = conditioned medium = filter-sterilized medium that BMS suspension cells had been growing in), poured into a 100 x 20 mm Petri plate, diluted with an equal volume of MS4D+8M, 2.4% SP (Sea Plaque agarose, FMC BioProducts, Rockland, ME) at about 37°C, and swirled to evenly disperse protoplasts. After the medium had solidified, the Petri plates were sealed with parafilm, placed in plastic storage boxes, and incubated in very dim light at about 26°C.

After 12 days, 12.5 ml of MS4D + 4% mannitol + 100 mg/l kanamycin was added to the plates, resulting in a final selective kanamycin concentration of about 50 mg/l. Nine days later, agarose slabs containing developing protoplast-derived colonies were replated onto Gel-rite-solidified MS4D + 100 mg/l kanamycin in 100 x 15 mm Petri plates. Kanamycin-resistant calli developed within 3 weeks from cell treatments except the "Control." The kanamycin-resistant calli were transferred individually to fresh Gel-rite solidified MS4D + 100 mg/l kanamycin, and were

subsequently maintained by transferring every 2 to 3 weeks onto medium of the same composition.

After selection on kanamycin, no "Control" calli remained, and three "Fusion-Low" calli, seven "Fusion-High" calli, and 169 "BTT" calli had survived.

14.2 Assay of insecticidal protein. Presence of the introduced DNA sequences was analyzed by Southern blot hybridization, using nick-translated NPT2 and insecticide gene DNA fragments (IG) as probes. Expression of the introduced genes was assayed by ELISA, using anti-NPT2 and anti-*B. thuringiensis* var. *tenebrionis* crystal protein antisera (CP). Separate tissue samples from each cell line were used for each assay. The results of the ELISA assays and Southern blots on the "Fusion" calli are summarized in Table 10.

ELISA positive were obtained only from calli whose DNA had regions of homology to both the NPT2 and IG probes. The lack of complete correspondence between the NPT2 and CP ELISAs could be due to reasons such as differential sensitivity of the assays, occasional problems with protein extraction or protein stability, or chimeric cell lines.

Twenty-eight of the 169 "BTT" calli were assayed by ELISA on two different days (separate tissue samples), using the anti-insecticidal protein antiserum. The results are shown in Table 11. Nine out of 28 (32%) of the calli tested positive in at least one of the two assays. There were only three discrepancies between the two assays (calli 6-21, 6-32, and 21-12). In each case, the callus tested positive in Experiment 1 and negative in Experiment 2. The most likely explanation is lower sensitivity of the Experiment 1 assay compared to the Experiment 2 assay.

Example 15

This Example teaches the expression in tomato (*Lycopersicum esculentum*) of the full-length *Bacillus thuringiensis* insecticide gene under control of both viral and T-DNA promoters.

15.1 Leaf tissue transformation. The leaf tissue transformation protocol combined and modified the procedures of Bevan, M. *et al.* (1985) *EMBO J.* 4:1921-1926, and Horsch, R.F. *et al.* (1985) *Science* 227:1229-1231. Leaves from three- to five-week-old greenhouse-grown seedlings were used. These leaves were washed, surface-sterilized, brushed, cut into 5 mm sections, and placed on agar-solidified regeneration medium (MS salts [Murashige, T., F. Skoog (1962) *Physiol. Plant.* 15:473-497], 30 g/l sucrose, 100 mg/l thiamine, 50 mg/l nicotinic acid, 50 mg/l pyridoxine, 5 μM zeatin riboside, 3 μM 3-indole-acetyl-DL-aspartic acid, 9 g/l agar, pH 5.65-5.8) or moistened filter paper for 1 day before bacterial inoculation. It was important to keep leaves moist. There were noticeable increases in survival with the 24-hour culture period prior to bacterial inoculation. Overnight cultures of bacteria were grown in L broth on selective antibiotics and diluted about 1/10 (an OD at 595 nm of about 250) prior to use. Bacteria were applied to each leaf disc with a sterile paintbrush and then plates were placed at 25°C for 2-3 days. After the 2 days, leaf discs were rinsed in antibiotic-containing regeneration media and then placed on antibiotic-containing regeneration agar plates. (All plates had an antibiotic-containing medium, containing 50 mg/l cefotoxin, or 250 mg/l carbenicillin and 50 mg/l vancomycin.)

Leaves transformed by bacterial strains containing the kanamycin resistance genes were grown under selection pressure using three procedures (Colbere-Garapin, F. *et al.* [1981] *J. Mol. Biol.* 15:1-14): (1) placement of inoculated explant on antibiotic-containing medium with 20 mg/l G418; (2) placement of explants on antibiotic medium with 5 mg/l G418; and (3) placement of explants on antibiotic medium with no G418. All treatments were transferred to plates containing about 15 to 20 mg/l G418 after approximately 5-7 days. An additional transfer two weeks later helped to eliminate further escapes. Tissues were then tested for opines.

15.2 Hypocotyl transformation. The use of inverted stem segments for *in vitro* transformation of tobacco tissues was first developed by Braun, A.C. (1956) *Cancer Res.* 16:53-56. The key to success using this method appears to be the fact that

wound cell divisions and callus formation occur at the basal end of the stem or hypocotyl segment. These divisions are important for two reasons. First, wound responses are a known requirement for transformation by *Agrobacterium tumefaciens*. Second, and more important, is that the subsequent cell divisions increase the numbers of transformed cells to a point where they can be screened for by opine analysis and survive heavy kanamycin selection pressure. The standard protocol used was to isolate hypocotyl segments (from sterile plants grown in growth incubator), to invert these so that the basal end was up, and then to inoculate with 2 μl bacteria applied with a micropipet (Pipetman). Bacteria were grown in L broth of MSSP medium supplemented with 50 μM acetosyrigone (Stachel, S.E., *et al.* [1985] *Nature* 318:624-629). After 1 to 2 days, the segment was washed thoroughly with liquid medium containing antibiotic and transferred to solidified antibiotic-containing medium. After visible callus was observed, it was excised, and cut into small pieces (about 3 mm) and subcultured on agar-solidified medium containing antibiotics. (These antibiotics included carbenicillin 260 mg/l and vancomycin 50 mg/l and G418 for selection pressure.) After tissues were grown to a large enough size (about 50 to 100 mg), they were subcultured and analyzed for opine content.

15.3 Transformations with *Agrobacterium* strains. Strains used for transformation of tomato were *A. tumefaciens* LBA4404 (Ooms, G. *et al.* [1981] *Gene* 41:33-50) containing the binary vectors pH450, pH575, pH576, pH578, pH582, and pH585. Transformations were made as described above on both leaf and hypocotyl tissues of UC82, LA14, V7 (LA14 X LA159) and V7R (LA159 X LA14) hybrids (carrying several marked genes in a heterozygous state), and proprietary hybrids 6-16, 6t0016, and 6t0045 M. Hypocotyl transformations generally yielded higher efficiency of transformation as detected by octopine production and kanamycin resistance. Efficiency of leaf transformations varied in the range of about 0.5% to about 5.0% depending on the particular *Agrobacterium* strains while hypocotyl transformations ranged in efficiency from about 5 to about 40% (Table 12).

Tomato plants were regenerated as described by Tatchell, S., A. Bins (1986) *Tomato Genet. Coop. Rept.* No. 36, pp. 35-36.

15.4 ELISAs. ELISA protocols were based on those described in Example 12.8. Techniques for preparing tissue samples were modified to enhance detection of insecticidal protein in plant tissues. Since in plant extracts, solubilization can be incomplete, this centrifugation step was eliminated. Leaf tissue was harvested from greenhouse plants, weighed, and placed in Eppendorf tubes on ice. Samples were quickly frozen in liquid nitrogen, thawed briefly, ground with a glass rod, and resuspended in 5X W/V PBS with 0.1 mM phenylmethylsulfonyl fluoride (PMSF). These samples are placed in the cold for about twenty minutes before taking aliquots for protein assays. Modified double antibody sandwich ELISAs with NADP-enhanced alkaline phosphatase were performed, tissue extracts containing 0.2 to 0.5 mg/ml protein (Clark, M.F., M. Bar-Joseph [1984] *Meth. Virol.* 7:51-85). Polystyrene microtiter plates were coated with mouse polyclonal antisera in 15 mM sodium carbonate buffer, 35 mM sodium bicarbonate, and pH 9.6 (0.1 ml/well) and stored at 4°C for 1 day to 2 weeks. Between each step, plates were washed 3 times with PBS-Tween (PBS + 0.05% "TWEEN"). Plates were blocked with blocking solution (PBS + "TWEEN" + 1% bovine serum albumin (BSA) fraction V (Sigma) and 1% casein acid hydrolysate (Sigma)). Plates were washed again and 0.1 ml/well antigen solution was added and incubated for about 2 to 3 hours at 25°C. Primary rabbit antisera against *B. thuringiensis* insecticidal protein were added to washed plates and incubated overnight at 4°C. The following morning, plates were washed and a 1:2500 dilution of alkaline phosphatase-labeled goat anti-rabbit antibody (Kirkegaard Perry Laboratories, Inc.) was added for about 2 hours. Plates were developed with modified nicotinamide adenine dinucleotide phosphate (NADP) enhancement (Johansson, A. *et al.* [1986] *J. Immun. Meth.* 87:7-11; Stanley, C.J. *et al.* [1985] *J. Immun. Meth.* 83:89-95; Self, C.H. [1985] *J. Immun. Meth.* 76:389-393). This involved addition of 0.1 ml/well of 300 mM NADP in diethanolamine substrate buffer (50 mM diethanolamine HCl pH 9.5, 1 mM $MgCl_2$) followed 25 minutes later by addition of

amplifier buffer (0.05 M sodium phosphate buffer, pH 7.2, containing 5 mg/ml BSA and 4% ethanol) with 0.55 mM *p*-iodonitrotetrazolium (Sigma), 1.5 mg/ml "DIAPHORASE" (Boehringer Mannheim), and 2 mg/ml alcohol dehydrogenase (Sigma). The reaction was stopped by the addition of 0.2 M H_2SO_4. Absorbances were read at 492 nm. Alternate development used was conventional *p*-nitrophenyl phosphate development.

15.5 Western blots. *Bacillus thuringiensis* insecticidal protein was detected in transformed tomato plants using protein immunoblot procedures ("Westerns"). The basic protocol involved preparation of leaf tissue by freezing in liquid nitrogen, grinding with mortar and pestle, and precipitating protein with 10% trichloroacetic acid. The samples were incubated on ice for at least 30 minutes and then spun at approximately 9000 rpm for 10 minutes. The pellet was resuspended in phosphate-buffered saline (PBS: 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4), and then insecticidal protein was precipitated in 20% ammonium sulfate, saturated ammonium sulfate being added slowly to samples at 4°C. Samples were then spun for 10 minutes at 9000 rpm, pellets were resuspended in modified PBS (PBS, 1% polyvinyl pyrrolidone, 0.006% 2-mercaptoethanol (2ME), 0.01% PMSF) and then dialyzed in PBS overnight at 4°C. Samples were cleared through glass wool and frozen at −20°C in cracking buffer (5% SDS, 6 M urea, 20 mM NaH_2PO_4, 0.02% bromophenol blue, 20% glycerol, and 0.3% 2ME). Tissue samples were then loaded on 8.5% acrylamide stacking gel (Laemmli, U.K. [1970] *Nature* 227:680-685). The stacking gel was run at 60 volts for about 1 hour and the running gel at 120 volts for 2 hours. Proteins were transferred to nitrocellulose (BA85) in Towbin's buffer (Towbin, H. *et al.* [1979] *Proc. Natl. Acad. Sci. USA* 76:4350-4354) in an IDEA Scientific GENIE Blotter at about 1 A and about 24 V for about 1 to 2 hours at 4°C. The nitrocellulose was briefly fixed in Towbin's buffer and then blocked for 1 hour at room temperature in Tris-buffered saline (TBS: 50 mM Tris-HCl, 0.15 M NaCl, pH 7.5) with 5% nonfat, dry mild (NFDM; Carnation). After blocking, it was washed 3 times (20 minutes each) with TBS + 0.5% NFDM + 0.5% Nonidet P-40 (detergent) before incubating

overnight at 4°C with anti-insecticidal protein antibodies. After primary antibody infection, the nitrocellulose was again washed 3 times (20 minutes each) with TBS + 0.5% NFDM + 0.5% sodium dodecyl sulfate (SDS). It was then incubated with goat anti-rabbit antibody alkaline phosphatase labeled (1:4000) for 2 hours at room temperature. Again, the nitrocellulose was washed for 20 minutes in TBS + 0.5% NFDM + 0.5% SDS, followed by 2 additional washes (10 minutes each) with TBS + 0.5% NFDM + 0.05% "TWEEN." The nitrocellulose was rinsed quickly three times in $MgCl_2$ buffer (0.1 M Tris HCl, pH 8.8, 0.1 M NaCl, and 5 mM $MgCl_2$) and then developed with 0.15 mg/ml 5-bromo-4-chloroinoxylphosphate/nitroblue tetrazolium substrate (Knecht, D.A., R.L. Dimond [1984] *Anal. Biochem.* 136:180-184; Blake, M.S. *et al.* [1984] *Anal. Biochem.* 136:175-179). Extracts of control tissues were spiked with protoxin and/or toxin during extraction to estimate recovery during the Western procedure. This showed that often insecticidal protein did not completely solubilize. As little as 0.01 ng of toxin was routinely detected on the blot. In 'spiked' samples, about 10 ng of standard was added to typical UC82 extract (from 100 mg tissue); the resultant signal on the blot represented about 30% recovery.

15.6 Bioassays. Bioassays were the most sensitive assay for the detection of *Bacillus thuringiensis* insecticidal protein. Two types of bioassays were conducted on putative transgenic plants. In the first, leaves were excised and placed in Petri plates containing moistened filter paper. A predetermined number of first instar *Manduca sexta* (tobacco hornworm) larvae were then added. This assay facilitated observations on *Manduca* and enabled larger numbers of samples to be tested. Substantial differences were observed between some varieties. However, results on a given R_0 or R_1 plant were not always consistent, due to either instability or insecticidal protein in excised leaves, or possible feeding of worms on moistened filter paper. In the second type of bioassay, first instar or, preferably, neonatal hornworms just hatched from eggs (eggs from Carolina Biological Supply) were placed on plants in the greenhouse, and checked daily for growth and mortality after 7 to 9 days. Problems with this assay included worms leaving tomato plants and dying of desiccation due to

high greenhouse temperatures ("wandering worms") and difficulties in finding hornworms on the tomato plants. These problems were adjusted for with control plant data, in which less than 20% mortality was consistently observed. "Wandering worms" were further controlled by placing the plants and worms in netted cages.

15.7 Nucleic acid analysis. Southern and Northern blot analyses were done using standard techniques well known to the art.

15.8 Results. R_0 plants were regenerated from pH450, pH576, pH577, pH578, pH582, and pH585 transformations and R_1 plants from pH450, pH576, and pH578 transformations (see Table 13). Kanamycin-resistant tissues were obtained from all transformations. Bud initials existed on transformations of most vectors (Table 12). R_0 plants from both pH578 and pH577 transformations that have been selected on 40 mg/l geneticin (equivalent to 400 mg/l kanamycin) gave high octopine signals.

Initially, many R_0 plants from transformations with pH450 were infertile due to long-term tissue culture. This was evident from increased flowering, and fruit set was observed in R_0 plants from pH578 and pH577 transformations, which were cultured for less time. In addition, cuttings from some older R_0 plants from pH450 transformations also showed improved fruit set. This improvement may also be in response to improved greenhouse conditions.

Two independent pH450 hypocotyl transformants yielded octopine-positive, G418-resistant tissues that regenerated plants containing octopine and *B. thuringiensis* insecticidal protein. Results from ELISA assays indicated insecticidal protein at levels ranging from 0.6 μg/ml to 2.1 μg/g total protein. A substantial decrease in both total protein in the leaf and in insecticidal protein (μg/g total protein) was associated with plant age. Substantial degradation occurred, and in Western blots all ELISA-positive samples show smearing starting at about 110 kD.

ELISAs were used to screen putative transformants for high expression of *Bacillus thuringiensis* insecticidal protein. Detectable levels (greater than 5 ng insecticidal protein per g leaf tissue) were observed in transformed plants from

pH450, pH577, and pH578, and in R_1 plants of pH577 and pH582 transformations. All ELISA-positive plants tested killed some *Manduca sexta* larvae in bioassays (Table 13).

Western blots were used to determine the form of insecticidal protein expressed (protoxin or toxin) and to quantify the level of expression more precisely. In pH450 transformants, which contain the full-length *Bacillus thuringiensis* insecticidal protein structural gene, both protoxin and toxin were observed in leaf tissues. Tissues from pH577 and pH578 transformed plants also had both forms of the insecticidal protein. Different forms of insecticidal protein (protoxin and toxin) were also observed in different regenerated plants from what initially appeared to be a single transformation event.

Results for many of the plants assayed by bioassay are listed in Table 13. Further analysis (not shown) by analysis of variance (ANOV) and Duncan's multiple range test showed significant differences at the 1% level. Transformed plants from pH450 and pH577 gave the best biological activity. Decreases in biological activity of transformed plants as a function of plant and leaf age were also observed. Control plants (UC82 R_0 plants and UC82 plants grown from seed) all gave low rates of mortality (less than 25%). It was significant that on several of the R_1 plants with lower *Manduca* mortality, the worm weights were significantly lower than controls. In addition, *M. sexta* larvae that failed to die immediately on *B. thuringiensis* insecticidal protein-containing plants also failed to grow at normal growth rates. These differences were clearly apparent after 2 weeks.

Southern analysis showed that insertions of T-DNA containing the plant-expressible insecticide gene were present in the expected organization, and are usually present in low copy number (about 1 to 2 copies/haploid genome). In all plants positive for protein and in bioassay positive plants tested, insecticidal protein sequences were observed in Northern blots of polyadenylated RNA. However, most of this RNA was shorter than expected.

The R_0 regenerated plants transformed by pH450 were marginally fertile. Southern, Northern, octopine (Aeerts, M. *et al.* [1979] *Plant Sci. Lett.* 17:43-50), and protein analysis indicated that the insecticide gene and flanking vector sequences were inherited in Mendelian fashion. Moreover, R_1 progeny plants gave positive results in bioassay, demonstrating that the capacity of the transformed plants to affect *Manduca sexta* is not restricted to regenerated plants.

Both bioassays and ELISAs of kanamycin-resistant pH577 and pH578 transformants indicated presence of insecticidal protein (Table 11). The pH578 regenerates were clearly transgenic plants, as indicated by octopine assays as well as kanamycin or G418 resistance. However, the inability to detect insecticidal protein in some transformants could be due to several reasons, e.g., a lack of assay sensitivity.

Example 16 – Potato Transformation Procedure

16.1 Plant material. Virus-free *in vitro* potato (*Solanum tuberosum*) plantlets of cultivars Kennebec, Superior, Atlantic, centennial Russet and Russet Norkotah were obtained from the University of Wisconsin, Department of Plant Pathology, Madison, WI. Russet Burbank Virus-free plantlets were obtained from the Potato Virus Research Laboratory at Colorado State University, Ft. Collins, CO. Hybrid Homestead seeds were purchased from Park Seed Company, Greenwood, SC. Russet Norkotah tubers were purchased from Deircks and Sons Inc., White Lake, WI.

16.2 Bacterial strains. *Agrobacterium tumefaciens* LBA4404, EHA101s, and Z707s strains were used for potato tissue infection. Gene constructs in these strains were pH615, pH619, pH623, pH624, and pH627. Bacteria was grown on YEP media with 250 mg/l streptomycin and 200 mg/l erythromycin for 24 hours at 28°C then suspended in MS liquid media prior to tissue inoculation.

16.3 Tissue culture media. Shoot induction medium consisted of MS salts (Murashige and Skoog, *supra*), 1.0 mg/l thiamine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 100 mg/l myo-inositol and 3% sucrose supplemented with 0.87 mg/l IAA

aspartic acid (Research Organics) and 1.76 mg/l zeatin riboside (Sigma). Hormones were added prior to autoclaving. The pH was adjusted to 5.8 with 0.1 M KOH and the medium was solidified with 7 g/l Tonomeko agar. Shoot induction medium containing carbenicillin at 500 mg/l and kanamycin at 25 mg/l was used for selection of transformed shoots. Stock plantlets were grown on MS basal medium without hormones in magenta GA7 containers (Magenta Corp.) and subcultured every 4 weeks. Shoot elongation and rooting medium consisted of MS basal medium with 25 mg/l kanamycin.

16.4 Procedure. Both stem and tuber tissue were used for transformation. Tubers were peeled and surface-sterilized in 10% liquid bleach for 15 minutes under constant agitation. A 0.7 cm diameter cork borer was used to make cores through the tuber. These cores were then sectioned into 2 to 3 mm thick discs and then inoculated (Sheerman, S., M.B. Bevan [1988] *Plant Cell Rep.* 7:13-16). Stem sections approximately 0.5 cm were made from *in vitro* grown stock plants 2 to 3 weeks after subculture. All sections containing axillary buds were discarded. Stem or tuber tissues were immediately soaked in 10^6, 10^7, or 10^8 ml^{-1} *Agrobacterium tumefaciens* cells for 5 to 10 minutes. They were then placed on sterile filter paper discs over *Nicotiana tabacum* feeder layers (Horsch, R.B. *et al.* [1985] *Science* 227:1229-1231) over shoot induction medium without antibiotics. They were cocultivated for 2 days then rinsed in liquid MS media containing 500 mg/l carbenicillin, blotted on sterile cardboard, and transferred to shoot induction media with carbenicillin 500 mg/l. After 4 to 5 days the stem segments or tuber discs were placed on shoot induction media containing 25 mg/l kanamycin and 500 mg/l carbenicillin. Cultures were transferred every 3 weeks. Buds began to appear in 4 to 6 weeks and continued to form for several weeks thereafter. Shoots were excised and rooted on MS media with 25 mg/l kanamycin, then tested for gene expression.

16.5 Results. Thirty-three Russet Burbank plants regenerated from tissues inoculated with *A. tumefaciens* (pH615) were selected on 25 mg/l kanamycin. Three of these plants were tested for expression of *B. thuringiensis* insecticidal protein by

ELISA; two were negative and one was positive. The insecticidal protein was expressed at a level of 5 ng/mg total protein. This plant also tested positive for NPT2 by ELISA. The plant DNA was then assayed by Southern analysis and tested positive for both insecticidal protein and NPT2. The DNA was also probed with a small section of the bacterial DNA (*vir*) to test for *Agrobacterium* contamination; none was present. A total of 7 Hybrid Homestead plants resulting from inoculations with pH624 and 4 Russet Burbank plants from inoculations with the pH627 have now been selected on kanamycin. Nine Kennebec plants regenerated after inoculations with the pH623 construct have also been selected.

Example 17 – Cotton Transformation

Agrobacterium tumefaciens strain LBA4404 (Hoekema, A. *et al.* [1983] *Nature* 303:179-180) carrying a binary vector (either pH576, pH577, pH578, pH582, or pH585) was cultured on YEP (10 m/l yeast extract, 10 g/l peptone, 5 g/l NaCl) medium containing 250 μg/ml streptomycin and 25 μg/ml kanamycin (both from Sigma) for selection and solidified with agar. Bacteria were scraped off the agar medium, suspended in a G_2 medium (MS salts (Bibco); Murashige and Skoog, *supra*, 100 mg/l myo-inositol, 0.4 mg/l thiamine·HCl, 5 mg/l 2iP, 0.1 mg/l NAA (all from Sigma), 30 g/l glucose, pH 5.9) to a concentration of about 10^8 cells/ml and were used for inoculation of cotyledon segments.

Cotton was transformed essentially as disclosed by Firoozabady, E. *et al.* (1987) *Plant Mol. Biol.* 10:105-116, and Firoozabady, E., U.S. Patent Application Serial No. 07/076,339. Cotyledon pieces (approximately 0.5 cm^2 surface area) from sterile 12 to 14 day old coker 201 seedlings were dipped in *A. tumefaciens* liquid cultures in Petri dishes and gently shaken for a few seconds to ensure contact of all cotyledon edges with the bacterial cultures. The cotyledon pieces were then blotted dry and plated on Whatman #1 filter paper on a callus initiation G_2 medium containing 0.2% "GEL-RITE" (Kelco). After three days cocultivation at low temperature (25°C) and 16 h/day photoperiod at 90 $\mu E \cdot m^{-1} \cdot s^{-1}$ light, cotyledon

pieces were transferred to Petri plates without the filter paper containing the same medium supplanted with 500 mg/l carbenicillin and 25 mg/l kanamycin sulfate (both from U.S. Biochemicals).

After 7-10 days of incubation, cotyledon pieces initiated transformed kanamycin-resistant microcalli (0.5 mm) at wound sites, while no callus from control untreated tissues or from tissues treated with LBA4404 grew on kanamycin. Two to three weeks later, 2-4 mm calli excised from original explants and transferred to fresh medium and incubated at 10 $\mu E \cdot m^{-1} \cdot s^{-1}$ light. Most calli (90-95%) were kanamycin resistant, and most kanamycin-resistant calli (75-90%) were positive when tested for octopine. Calli were placed on embryogenic callus induction medium G_3 (G_3 = G_2 but with 0.1 mg/l 2iP and 5 mg/l NAA) for two weeks and then placed and maintained on embryogenic medium G_0 (same as G_2 with no hormones) under selection. Mature somatic embryos were transferred to lower ionic strength medium, GRM_{gn} (modified from the medium of Stewart, J.M., C.L. Hsu [1977] *Planta* 137:113-177, by the addition of 0.01 mg/l NAA, and 0.1 mg/l GA, and use of 5 g/l glucose instead of sucrose). Plants were shown to be transformed by their resistance to kanamycin by production of callus from leaf tissue in the presence of kanamycin, production of octopine, ELISA for NPT2 and by DNA hybridization, and Western immunoblot analyses. Plants were transferred to soil for further analysis. The whole process from infection until transgenic plants were transferred to soil took about 6 to 8 months.

Table 1. Insects susceptible to *B. thuringiensis* insecticidal protein

COLEOPTERA
Popillia japonica (Japanese beetle)
Sitophilus granarius (granary weevil)
DIPTERA
Aedes aegypti (yellow-fever mosquito)
A. atlanticus
A. cantans
A. capsius
A. cinereus
A. communis
A. detritus
A. dorsalis
A. dupreei
A. melanimon
A. nigromaculis (pasture mosquito)
A. punctor
A. sierrensis (western treehole mosquito)
A. sollicitans (brown salt marsh mosquito)
Aedes sp.
A. taeniorhynchus (black salt marsh mosquito)
A. tarsalis
A. tormentor
A. triseriatus
A. vexans (inland floodwater mosquito)
Anopheles crucians
A. freeborni
A. quadrimaculatus (common malaria mosquito)
A. sergentii
A. stephensi
Anopheles sp.
Chironomus plumosus (*Chironomus*: midges, biting)
Chironomus sp.
C. tummi
Culex erraticus
C. inornata
C. nigripalus
C. peus
C. pipiens (northern house mosquito)
C. quinquefasciatus (*C. pipiens fatigans*) (southern house mosquito)
C. restuans
Culex sp.
C. tritaeniorhynchus

C. tarsalis (western encephalitis mosquito)
C. territans
C. univittatus
Culiseta incidens (*Culiseta*: mosquitos)
C. inornata
Diamessa sp.
Dixa sp. (*Dixa*: midges)
Eusimulium (*Simulium*) *latipes* (*Eusimulium*: gnats)
Goeldichironomus holoprasinus
Haematobia irritans (horn fly)
Hippelates collusor
Odagmia ornata
Pales pavida
Polpomyia sp. (*Polpomyia*: midges, biting)
Polypedilum sp. (*Polypedilum*: midges)
Psorophora ciliata
P. columiae (*confinnis*) (Florida glades mosquito, dark rice field mosquito)
P. ferox
Simulium alcocki (*Simulium*: black flies)
S. argus
S. cervicornatum
S. damnosum
S. jenningsi
S. piperi
S. tescorum
S. tuberosum
S. unicornatum
S. venustum
S. verecundum
S. vittatum
Uranotaenia inguiculata
U. lowii
Wyeomyia mitchellii (*Wyeomyia*: mosquitos)
W. vanduzeei

HYMENOPTERA

Athalia rosae (as *colibri*)
Nematus (*Pteronidea*) *ribesii* (imported currantworm)
Neodiprion banksianae (jack-pine sawfly)
Priophorus tristis
Pristiphora erichsonii (larch sawfly)

LEPIDOPTERA

Achaea janata
Achroia grisella (lesser wax moth)
Achyra rantalis

Acleris variana (black-headed budworm)
Acrobasis sp.
Acrolepia alliella
Acrolepiopsis (*Acrolepia*) *assectella*
Adoxophyes orana (apple leaf roller)
Aegeria (*Sanninoidea*) *exitiosa* (peach tree borer)
Aglais urticae
Agriopsis (*Erannis*) *aurantiaria* (*Erannis*: loopers)
A. (*E.*) *leucophaeria*
A. marginaria
Agrotis ipsilon (as *ypsilon*) (black cutworm)
A. segetum
Alabama argillacea (cotton leafworm)
Alsophila aescularia
A. pometaria (fall cankerworm)
Amorbia essigana
Anadevidia (*Plusia*) *peponis*
Anisota senatoria (orange-striped oakworm)
Anomis flava
A. (*Cosmophila*) *sabulifera*
Antheraea pernyi
Anticarsia gemmatalis (velvetbean caterpillar)
Apocheima (*Biston*) *hispidaria*
A. pilosaria (*pedaria*)
Aporia crataegi (black-veined whitemoth)
Archips argyrospilus (fruit-tree leaf roller)
A. cerasivorana (ugly-nest caterpillar)
A. crataegana
A. podana
A. (*Cacoecia*) *rosana*
A. xylosteana
Arctia caja
Argyrotaenia mariana (gray-banded leaf roller)
A. velutinana (red-banded leaf roller)
Ascia (*Pieris*) *monuste orseis*
Ascotis selenaria
Atteva aurea (alianthus webworm)
Autographa californica (alfalfa looper)
A. (*Plusia*) *gamma*
A. nigrisigna
Autoplusia egena (bean leaf skeletonizer)
Azochis gripusalis
Bissetia steniella
Bombyx mori (silkworm)

Brachionycha sphinx
Bucculatrix thurberiella (cotton leaf perforator)
Bupolus pinarius (*Bupolus*: looper)
Cacoecimorpha pronubana
Cactoblastis cactorum
Caloptilia (*Gracillaria*) *invariabilis*
C. (*G.*) *syringella* (lilac leaf miner)
C. (*G.*) *theivora*
Canephora asiatica
Carposina niponensis
Ceramidia sp.
Cerapteryx graminis
Chilo auricilius
C. sacchariphagus indicus
C. suppressalis (rice stem borer)
Choristoneura fumiferana (spruce budworm)
C. murinana (fir-shoot roller)
Chrysodeixis (*Plusia*) *chalcites*
Clepsis spectrana
Cnaphalocrocis medinalis
Coleotechnites (*Recurvia*) *milleri* (lodgepole needle miner)
C. nanella
Colias eurytheme (alfalfa caterpillar)
C. lesbia
Colotois pennaria
Crambus bonifatellus (fawn-colored lawn moth, sod webworm)
C. sperryellus
Crambus sp.
Cryptoblabes gnidiella
Cydia funebrana
C. (*Grapholitha*) *molesta* (oriental fruit moth)
C. (*Laspeyresta*) *pomonella* (codling moth)
Datana integerrima (walnut caterpillar)
D. ministra (yellow-necked caterpillar)
Dendrolimus pini
D. sibiricus
Depressaria marcella (a webworm)
Desmia funeralis (grape leaf folder)
Diachrysia (*Plusia*) *orichalcea* (a semilooper)
Diacrisia virginica (yellow woolybear)
Diaphania (*Margaronia*) *indica*
D. nitidalis (pickleworm)
Diaphora mendica
Diatraea grandiosella (southwestern corn borer)

D. saccharalis (sugarcane borer)
Dichomeris marginella (juniper webworm)
Drymonia ruficornis (as *chaonia*)
Drymonia sp.
Dryocampa rubicunda
Earias insulana
Ectropis (*Boarmia*) *crepuscularia*
Ennomos subsignaria (elm spanworm)
Elphestia (*Cardra*) *cautella* (almond moth)
E. elutella (tobacco moth)
E. (*Anagasta*) *kuehniella* (Mediterranean flour moth)
Elpinotia tsugana (a skeletonizer)
Epiphyas postvittana
Erannis defoliaria (mottled umber moth)
E. tiliaria (linden looper)
Erinnysis ello
Eriogaster henkei
E. lanestris
Estigmene acrea (salt marsh caterpillar)
Eublemma amabilis
Euphydryas chalcedona
Eupoecilia ambiguella
Euproctis chrysorrhoea (*Nygmi phaeorrhoea*) (browntail moth)
E. fraterna
E. pseudoconspersa
Eupterote fabia
Eutroula (*Simaethis*) *pariana*
Euxoa messoria (dark-sided cutworm)
Galleria mellonella (greater wax moths)
Gastropacha quercifolia
Halysidota argentata
H. caryae (hickory tussock moth)
Harrisina brillians (western grape skeletonizer)
Hedya nubiferana (fruit tree tortrix moth)
Heliothis (*Helicoverpa*) *armigera* (*Heliothis* = *Chloridea*) (gram pod borer)
H. (*H.*) *assulta*
Heliothis petigera
H. virescens (tobacco budworm)
H. viriplaca
H. zea (cotton bollworm, corn earworm, soybean podworm, tomato fruitworm, sorghum headworm etc.)
Hellula undalis (cabbage webworm)
Herpetogramma phaeopteralis
Heterocampa guttivitti (saddled prominent)

H. manteo (variable oak leaf caterpillar)
Holcocera pulverea
Homoeosoma electellum (sunflower moth)
Homona magnanima
Hyloicus pinastri
Hypeuryntis coricopa
Hyphantria cunea (fall webworm)
Hypogymna morio
Itame (*Thamnonoma*) *wauria* (a spanworm)
Junonia coenia (buckeye caterpillars)
Kakivoria flavofasciata
Keiferia (*Gnorimoschema*) *lycopersicella* (tomato pinworm)
Lacanobia (*Polia*) *oleracea*
Lambdina athasaria pellucidaria
L. fiscellaria fiscellaria (hemlock looper)
L. fiscellaria lugubrosa
L. fiscelleria somniaria
Lampides boeticus
Leucoma (*Stilpnotia*) *salicis* (satin moth)
L. wiltshirei
Lobesia (= *Polychrosis*) *botrana*
Loxostege commixtalis (alfalfa webworm)
L. sticticallis (beet webworm)
Lymantria (*Porthetria*) *dispar* (gypsy moth) (*Lymantria*: tussock moth)
L. monacha (nun-moth caterpillar)
Malacosoma americanum (eastern tent caterpillar)
M. disstria (forest tent caterpillar)
M. fragilis (= *fragile*) (Great Basin tent caterpillar)
M. neustria (tent caterpillar, lackey moth)
M. neustria var. *testacea*
M. pluviale (western tent caterpillar)
Mamestra brassicae (cabbage moth)
Manduca (*Intoparce*) *quinquemaculata* (tomato hornworm)
M. (*I.*) *sexta* (tobacco hornworm)
Maruca testulalis
Melanolophia imitata
Mesographe forficalis
Mocis repanda (*Mocis*: semilooper)
Molippa sabina
Monema flavescens
Mythimna (*Pseudaletia*) *unipuncta* (armyworm)
Nephantis serinopa
Noctua (*Triphaena*) *pronuba*
Nomophila noctuella (lucerne moth)

Nymphalis antiopa (mourning-cloak butterfly)
Oiketicus moyanoi
Ommatopteryx texana
Operophtera brumata (winter moth)
Opsophanes sp.
O. fagata
Orgyia (*Hemerocampa*) *antiqua*
O. leucostigma (white-marked tussock moth)
O. (*H.*) *pseudotusgata* (Douglas-fir tussock moth)
O. thyellina
Orthosia gothica
Ostrinia (*Pyrausta*) *nubialis* (European corn borer)
Paleacrita vernata (spring cankerworm)
Pammene juliana
Pandemis dumetana
P. pyrusana
Panolis flammea
Papilio cresphontes (orangedog)
P. demoleus
P. philenor
Paralipsa (*Aphemia*) *gularis*
Paralobesia viteana
Paramyelosis transitella
Parnara guttata
Pectinophora gossypiella (pink bollworm)
Pericallia ricini
Peridroma saucia (variegated cutworm)
Phalera bucephala
Phlogophora meticulosa
Phryganidia californica (California oakworm)
Phthorimaea (= *Gnorimoschema*) *operculella* (potato tuberworms)
Phyllonorycter (*Lithocolletis*) *blancardella*
Pieris brassicae (large white butterfly)
P. canidia sordida
P. rapae (imported cabbageworm, small white butterfly)
Plathypena scabra (green cloverworm)
Platynota sp.
P. stultana
Platyptilia carduidactyla (artichoke plume moth)
Plodia interpunctella (Indian-meal moth)
Plutella xylostella as *maculipennis* (diamondback moth)
Prays citri (citrus flower moth)
P. oleae (olive moth)
Pseudoplusia includens (soybean looper)

Pygaera anastomosis
Rachiplusia ou
Rhyacionia buoliana (European pine shoot moth)
Sabulodes caberata
Samia cynthia
Saturnia pavonia
Schizura concinna (red-humped caterpillar)
Schoenobius bipunctifer
Selenephera lunigera
Sesamia inferens
Sibine apicalis
Sitotroga cerealella (Angoumois grain moth)
Sparganothis pilleriana
Spilonota (*Tmetocera*) *ocellana* (eye-spotted budmoth)
Spilosoma lubricipeda (as *menthastri*)
S. virginica
Spilosoma sp.
Spodoptera (*Prodenia*) *eridania* (southern armyworm)
S. exigua (beet armyworm, lucerne caterpillar)
S. frugiperda
S. littoralis
S. litura
S. mauritia
S. (*P.*) *ornithogalli* (yellow-striped armyworm)
S. (*P.*) *praefica*
Syllepte derogata
S. silicalis
Symmerista canicosta
Thaumetopoea pityocampa (pine processionary caterpillar)
T. processionea
T. wauaria (currant webworm)
T. wilkinsoni
Thymelicus lineola (European skipper)
Thyridopteryx ephemeraeformis (bagworm)
Tineola bisselliella (webbing clothes moth)
Tortrix viridana (oak tortricid)
Trichoplusia ni (cabbage looper)
Udea profundalis (celery leaf tier)
U. rubigalis
Vanessa cardui (painted-lady)
V. io
Xanthopastis timais
Xestia (*Amathes, Agrotis*) *c-nigrum* (spotted cutworm)
Yponomeuta cognatella (= *Y. evonymi*) (*Yponomeuta* = *Hyponomeuta*)

Y. evonymella
Y. mahalebella
Y. malinella (small ermine moth)
Y. padella (small ermine moth)
Y. rorrella
Zeiraphera diniana
MALLOPHAGA
Bovicola bovis (cattle biting louse)
B. crassipes
B. limbata
B. ovis
Liperus caponis (wing louse)
Menacanthus stramineus
Menopon gallinae (shaft louse)
TRICHOPTERA
Hydropsyche pellucida
Potamophylax rotundipennis

Table 2. Plants recommended for protection by *B. thuringiensis* insecticidal protein

alfalfa	escarole	pomegranate
almonds	field corn	potatoes
apples	filberts	radishes
artichokes	flowers	rangeland
avocados	forage crops	raspberries
bananas	forest trees	safflower
beans	fruit trees	shade trees
beets	garlic	shingiku
blackberries	grapes	small grains
blueberries	hay	soybeans
broccoli	kale	spinach
brussels sprouts	kiwi	squash
cabbage	kohlrabi	stonefruits
caneberries	lentils	stored corn
carrots	lettuce	stored grains
cauliflower	melons	stored oilseeds
celery	mint	stored peanuts
chard	mustard greens	stored soybeans
cherries	nectarines	stored tobacco
Chinese cabbage	onions	strawberries
chrysanthemums	oranges	sugarbeets
citrus	ornamental trees	sugar maple
collards	parsley	sunflower
cos lettuce	pasture	sweet corn
cotton	peaches	sweet potatoes
cranberries	peanuts	tobacco
crop seed	pears	tomatoes
cucumbers	peas	turf
currants	pecans	turnip greens
dewberries	peppers	walnuts
eggplant	pome fruit	watermelons
endive		

Table 3. Varieties of *B. thuringiensis*

alesti
aizawai
canadensis
dakota
darmstadiensis
dendrolimus
entomocidus
finitimus
fowleri
galleriae
indiana
israelensis
kenyae
kurstaki
kyushuensis
morrisoni
ostriniae
pakistani
sotto
thompsoni
thuringiensis
tolworthi
toumanoffi
wuhanensis

Table 4. Index of plasmid and strains

Strain or Plasmid	Constructed or used in Example	See Figure	Made From (α comments)
A. tumefaciens	6		(ubiquitous)
A. rhizogenes	5		(also see background)
B. thuringiensis var. *kurstaki* HD-73	1.1	1	
ColE1	2.5		
E. coli GM33	2.3		
E. coli HB101	1.1		
E. coli JM103	2.1		
E. coli K802	2.2		
MBT3	3.3		M13mp8, p123/58-10
MBT3 (*Nco*)	3.4		MBT3
MBT14	3.3		M13mp8, p123/58-10
mWB2344	2.1		
M13-Bt-A	2.1		mWB2344, p123/58-10
M13-Bt-A(*Bam*)	2.1		M13-Bt-A
M13-Bt-S	2.1		mWB2344, p123/58-10
M13mp7	3.1		
M13mp8	3.3		
M13-PpBt	4.4		MBT3 (*Nco*), M13-3.8Ab
M13-1	3.1		M13mp7, pNS5
M13-3	3.1		M13mp7, pNS5
M13-3A/B18a	3.2		M13-3
M13-3.8A	4.1		M13mp7, 177.4
M13-3.8Aa	4.2		M-13-3.8Ac

Table 4 (continued)

Strain or Plasmid	Constructed or used in Example	See Figure	Made From (α comments)
M13-3.8Ab	4.3		M13-3.8Aa
M13-3.8Ac	4.2		M-13-3.8A
M13-3.8S	4.1		M13mp7, 177.4
pBR322	1.1		M13mp7, 177.4
pCF44	3.1		pBR322, pTiC58
pCF44A	3.1		pCF44
pKS-*pro*I	2.2	3	pKS111, = pTR-*pro*I
pKS-*pro*I(*Bam*)	2.2	2.2	pKS-*pro*I
pKS-4	2.5	2	pBR322, pRZ102
pKS111	2.2, 12.3	2, 3	pRK290, pTi15955
pKS111-K	4.5		pKS4 (prZ102), pKS111
pKS111-N	3.5		pCF44, pKS111-K
pKS111-NpBt	3.5		MBT3(*Nco*), M13-3A/B18a
pKS111-PpBt	4.5		M13-PpBt, pKS111-K
pNS5	3.1		pBR322, pCF44A
pPH1J1	9		
pRK290	2.2, 9		
pRK2013	9		
pRZ102	2.5		ColE1, Tn5
pTiA66	2.4		
pTi15955	2.4	2	
p8.8	4.1		pBR322, 177.4
p11-83a	2.3	3	pKS-*pro*I(*Bam*), pKS-4

Table 4 (continued)

Strain or Plasmid	Constructed or used in Example	See Figure	Made From (α comments)
p11-83b	2.3	3	p11-83a, M13-Bt-A(*Bam*)
p123/58-3	1.1	1	*B. thuringiensis* var. *kurstaki* HD-73, pBR322
p123/58-10	1.1	1	*B. thuringiensis* var. *kurstaki* HD-73, pBR322
p403	2.2	2	pBR322, pTi15955
"1.6"	2.2	2	(= transcript 24, see also Detailed Description)
177.4	4.1		Charon 24A, *P. vulgaris* cv. Tendergreen
pBt73-161	11.1		*B. thuringiensis* var. *kurstaki* HD-73, pBR322
pBt73-10 (*Bam*)	11.2		p123/58-10
pBt73-16	11.2	4	pBt73-10(*Bam*), pBt73-161
pTR-*pro*I	12.1	4	= pKS-*pro*I
pTR-*pro*I(*Bam*)	12.1	4	pTR-*pro*I
pBR325	12.3	4	
pBR325aBB	12.3	4	pBR325
p403B	12.1	4	pBR325aBB, pTR-*pro*I(*Bam*)
M13mp19	12.4	4	
1.6.4	12.4	4	M13mp19, pBt73-16
1.6.4B-3.8.3	12.4	4	1.6.4

Table 4 (continued)

Strain or Plasmid	Constructed or used in Example	See Figure	Made From (α comments)
p403B/BTBΔ3	12.3	4	1.6.4B-3.8.3, p403B
pH4-1	12.1		pSUP106, pTi15955, CaMV, Tn*5*
pH400	12.1		pH4-1
pDOB412	12.2		CaMV, pBR322
pDOB512	12.2		pDOB412
p403BRL1	12.3		p403B
pDOB514	12.3		pDOB513
pMAN514	12.3		p403BRL1, pDOB514
pKS4.2	12.3		pKS4
pKS4.3	12.3		pKS4.2
pBR322Bam	12.3		pBR322, *lambda*
p11-83c	12.3		pKS4.3, pBR322Bam$^-$
pCJ161	12.5		1.6.4B-3.8.3, pCG116
pH450	12.6		pCJ161, pH400
pCG116	12.3		p11-83c, pTR-*pro*I(*Bam*)

Table 5. Deposited strains

NRRL B-4488	*B. thuringiensis* var. *kurstaki* HD-73
NRRL B-15394	*E. coli* C600 (pKS-4)
NRRL B-11371	*E. coli* HB101
NRRL B-12014	*E. coli* RR1 (pBR322)
ATCC 37017	pBR322
ATCC 15955	*A. tumefaciens* (pTi15955)
NRRL B-15393	*E. coli* HB101 (p8.8)
NRRL B-15612	*E. coli* HB101 (p123/58-10)
NRRL B-15759	*E. coli* HB101 (pBt73-16)
NRRL B-18009	*E. coli* K802 (pH4-1)
NRRL B-15486	*E. coli* CSH52 (pSUP106)

Table 6.

MS Medium		
NH_4NO_3	1.65	g/l
KNO_3	1.9	g/l
$CaCl_2 \cdot 2H_2O$	440	mg/l
$MgSO_4 \cdot 7H_2O$	370	mg/l
KH_2PO_4	170	mg/l
KI	0.83	mg/l
H_3BO_3	6.2	mg/l
$MnSO_4 \cdot 4H_2O$	22.3	mg/l
$ZnSO_4 \cdot 7H_2O$	8.6	mg/l
$Na_2MoO_4 \cdot 2H_2O$	0.25	mg/l
$CuSO_4 \cdot 5H_2O$	0.025	mg/l
$CoCl_2 \cdot 6H_2O$	0.025	mg/l
$Na_2 \cdot EDTA$	37.23	mg/l
$FeSO_4 \cdot 7H_2O$	27.85	mg/l
Inositol	1	g/l
Nicotinic acid	50	mg/l
Pyroxidine·HCl	50	mg/l
Thiamine·HCl	50	mg/l
Sucrose	30	g/l
Agar	8	g/l

Table 7.

	Experiment 1[5]		Experiment 2[6]	
Clone	A_{492}[1]	CP[2]	A_{492}	CP
100	0.17 ± 0.03[7]	+	0.14	+
101	0.02 ± 0.01	–	0.07	±
102	0.06 ± 0.02	±	0.21	+
103	0.00 ± 0.00	–	ND	ND
104	ND[3]	ND	0.11	+
105	0.00 ± 0.00	–	0.10	+
106	0.10 ± 0.02	+	0.01	–
107	0.06 ± 0.03	±	0.06	–
109	0.11 ± 0.05	+	0.03	–
110	0.06 ± 0.01	±	0.13	+
111	0.12 ± 0.02	+	0.00	–
NX[4]	-0-		-0-	

[1]Average of three ELISA determinations. Absorbance at 492 nm, corrected by subtracting the value for the NX control.

[2]Rated as having (+) or not having (–) crystal protein. ± indicates a marginal rating.

[3]Not determined.

[4]Untransformed *Nicotiana tabacum* var. "Xanthi" control. In experiments 1 and 2, repectively, 18 and 19 control leaves obtained from different plants were averaged.

[5]Experiment 1 was standardized for equal tissue wet weight.

[6]Experiment 2 was standardized for equal plant protein concentrations.

[7]± standard error of the mean.

Table 8.

Clone number	Replicate number	Total larvae	Dead larvae
100	1	4	1
	2	5	0
	3	5	4
103	1	5	0
	2	5	0
	3	5	0
106	1	5	0
	2	5	0
	3	5	0
109	1	5	2
	2	5	0
	3	5	3
111	1	5	2
	2	5	1
	3	5	0

Table 9.

Clone number	Replicate number	Total larvae	Dead larvae
100	1	4	2
	2	4	3
	3	6	3
103	1	6	0
	2	6	2
	3	6	2
106	1	6	2
	2	8	3
	3	10	6
109	1	14	2
	2	6	1
	3	6	4
111	1	6	3
	2	6	2
	3	6	3

Table 10.

		ELISA Assays		Southern Blots	
			NPT2	IG	
Callus #*	Treatment	NPT2	Btt	Probe	Probe
5-1	Fusion-High	–	–	–	–
5-2	Fusion-High	+	+	+	+
5-3	Fusion-High	–	ND	ND	ND
19-1	Fusion-Low	–	+	ND	ND
20-1	Fusion-High	–	+	+	+
20-2	Fusion-High	+	–	+	+
20-3	Fusion-High	–	+	+	+
34-1	Fusion-Low	–	ND	+	+
35-1	Fusion-High	+	+	+	+
EG5	Neg. Control	–	–	–	–

ND = not determined

*Only 9 of the 10 kanamycin resistant "Fusion" calli produced sufficient callus tissue for these analyses.

Table 11.

Callus #	Exp. 1	Exp. 2
6-1	+	+
6-3	–	–
6-4	ND	–
6-9	+	ND
6-11	–	–
6-12	+	+
6-18	+	+
6-19	+	+
6-21	+	–
6-23	+	+
6-32	+	–
21-3	–	–
21-4	–	–
21-5	–	–
21-8	–	–
21-9	–	–
21-12	+	–
21-13	–	–
21-16	–	–
21-19	–	–
21-26	–	–
36-1	–	–
36-3	–	–
36-4	–	–
36-12	–	–
36-14	–	–
36-17	–	–
36-25	–	–

ND = not determined

Table 12. Transformation of *Lycopersicum esculentum* hypocotyl and leaf disc tissues with various binary vectors

	Hypocotyl Tissue		Leaf Tissue	
Transforming Plasmid	Percent Transformed	Percent Transformed w/buds	Percent Transformed	Percent Transformed w/buds
pH450	19 (178)[a]	9 (34)	0.17 (1189)	100 (2)
pH575	9 (180)	28 (17)	5 (640)	0
pH576	21 (123)	19 (26)	5 (1993)	0
pH577	11 (282)	22 (32)	1 (1430)	42 (12)
pH578	9 (136)	58 (12)	4 (820)	23 (31)
pH582	9 (97)	0		
pH585	11.5 (104)	25 (12)		

[a](number)

Table 13. Summary of bioassays and ELISAs on tomato

Plant*	Gen	Plant age months	Percent mortality (n)	% worm weight	ELISA
UC82	(F_1)	<6	13 (385)	100	–
UC82	(R_0)	<6	17 (50)	91	–
V7R	(F_1)	<6	23 (30)	118	–
V7R	(R_0)	<6	19 (11)	78	–
pH450-7	(R_0)	<6	73 (55)	77	+
		>6	39 (36)	86	+
pH450-13	(R_0)	<6	35 (20)	29	+
pH450-19	(R_0)	<6	70 (20)	32	+
pH450-1-1a	(R_1)	<6	50 (38)	20	+
pH450-2-3a	(R_1)	<6	17 (18)	91	–
pH450-2-5a	(R_1)	<6	10 (10)	55	–
pH450-2-5b	(R_1)	<6	22 (9)	38	–
pH450-4-1c	(R_1)	<6	78 (9)	69	+
pH450-4-2e	(R_1)	<6	78 (18)	40	+
		>6	46 (13)	120	+
pH450-4-2f	(R_1)	<6	50 (14)	32	+
pH577-3a	(R_0)	<6	74 (46)	19	+
pH577-3c	(R_0)	<6	80 (20)	15	+
pH577-3d	(R_0)	<6	40 (10)	39	NA
pH577-3g-1	(R_0)	<6	85 (20)	8	+
pH577-3g-2	(R_0)	<6	65 (30)	19	+
pH577-3g-4	(R_0)	<6	19 (16)	43	NA
pH577-3g-7	(R_0)	<6	60 (20)	20	NA
pH577-17-5	(R_0)	<6	38 (32)	61	+
pH577-17-6	(R_0)	<6	0 (10)	80	–
pH577-21b	(R_0)	<6	30 (10)	60	NA
pH578-6-1b	(R_0)	<6	65 (23)	89	+
pH578-6-2c	(R_0)	<6	0 (14)	65	–
pH578-15-10	(R_0)	<6	67 (30)	47	+

*All plants except UC82 and V7R are octopine positive and kanamycin resistant.
NA = not assayed.